UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

Produção de xilooligossacarídeos a partir de resíduos lignocelulósicos e fungos filamentosos

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Orientador: Prof. PhD. Marco Antônio Záchia Ayub
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Porto Alegre, 2018

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A banca examinadora, abaixo assinada, aprova a Tese "Produção de Xilooligossacarídeos a partir de resíduos lignocelulósicos e fungos filamentosos.", elaborada por Bruna da Silva Menezes, como pré-requisito para obtenção do grau de Doutor em Ciência e Tecnologia de Alimentos.

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RESUMO

Xilooligossacarídeos (XOS) são produzidos a partir materiais lignocelulósicos contendo xilanos através de métodos químicos, hidrólise enzimática direta de um substrato susceptível à ação de xilanases e outras enzimas líticas, ou uma combinação de tratamentos químicos e enzimáticos. Os XOS são reconhecidos por trazerem benefício a saúde e são considerados ingredientes prebióticos. A utilização de resíduos agro-industriais produzidos no Rio Grande do Sul, tais como a casca de arroz, a casca de soja e o extrato de malte proveniente de cervejarias tornam-se substratos com grande potencial para a produção da enzima xilanase, principalmente em cultivo em estado sólido. Neste trabalho, foram analisados o potencial de diversos fungos selvagens e de um fungo recombinante na produção da enzima xilanase e outras enzimas importantes para a hidrólise de biomassa lignocelulósica, e a aplicação destes microrganismos para a produção de XOS em cultivos em estado sólido. Através de uma seleção de fungos foi possível definir o *Aspergillus brasiliensis* BLf1 como maior produtor de xilanase em cultivo sobre casca de arroz, obtendo atividade de 120,5 $U.g^{-1}$ substrato. Nesta seleção foram analisadas também as enzimas celulase, β-glicosidase e β-xilosidase. Um planejamento experimental fracionário $2^{(5-1)}$ deste fungo selecionado e do fungo recombinante *Aspergillus nidulans* XynC A773 determinaram variáveis que influenciam a produção da enzima xilanase, chegando a uma atividade máxima de 230,7 $U.g^{-1}$ para *A. brasiliensis* BLf1 e 187,9 $U.g^{-1}$ para *A. nidulans* XynC A773. Posteriormente, estas preparações enzimáticas foram aplicadas à casca de arroz para hidrolisar sua estrutura hemicelulósica polimérica e obter xilolossacarídeos (37,25 mg de $XOS.g^{-1}$ de substrato e 75,92 mg de $XOS.g^{-1}$ de substrato, respectivamente). Por fim, foi avaliado o potencial prebiótico de XOS obtidos. Os resultados deste estudo revelaram o crescimento de *L. plantarum* BL011 e *B. lactis* BB-12 em XOS, com um aumento de massa celular seca de até 1,7 $g.L^{-1}$ em 120h. Os resultados obtidos neste trabalho sugerem que é possível a obtenção de XOS a partir de resíduos abundantes no Estado e que os mesmos possuem potencial para serem utilizados como prebióticos, podendo, portanto, ser usados em aplicações relacionadas aos alimentos funcionais.

Palavras-chaves: Resíduos lignocelulósicos, xilanase, xilooligossacarídeos (XOS), hidrólise enzimática, prebióticos.

ABSTRACT

Xylooligosaccharides (XOS) are produced from lignocellulosic materials containing xylan by chemical methods, direct enzymatic hydrolysis of susceptible substrates using xylanases and other lytic enzymes, or a combination of chemical and enzymatic treatments. XOS are recognized for bringing benefit to health of the host and are considered prebiotic ingredients. The use of lignocellulosic residues produced in Rio Grande do Sul, such as rice husk, soybean hulls, and spent malt from brewery hold potential for the production of xylanase enzyme, especially under solid-state cultivation. In this work, several wild strains of fungi and one recombinant strain were tested for their potential of producing xylanase and their application in solid-state cultivation to obtain XOS. It was possible to define the *Aspergillus brasiliensis* BLf1 as the best producer of xylanase on rice husk, obtaining an activity of 120.5 $U.g^{-1}$. Other important lytic enzymes were also analyzed: cellulase, β-glucosidase, and β-xylosidase. The statistical experiment fractional factorial design $2^{(5-1)}$ of cultures of this fungus and of the recombinant strain *Aspergillus nidulans* XynC A773 defined the variables that influenced the production of xylanase, showing maximal activities of 230.7 $U.g^{-1}$ for *A. brasiliensis* BLf1 and 187.9 $U.g^{-1}$ for *A. nidulans* XynC A773. Subsequently, these enzymatic preparations were applied to rice husk to hydrolyse its polymeric hemicellulosic structure and obtain xylooligosaccharides (37.25 mg $XOS.g^{-1}$ substrate and 75.92 mg $XOS.g^{-1}$ substrate, respectively). Finally, the prebiotic potential of XOS was evaluated by using them to grow *L. plantarum* BL011 and *B. lactis* BB-12, showing an increase in the dry cell mass of 1.7 $g.L^{-1}$ at 120 hours. The results obtained in this research suggest that it is possible to obtain XOS from agro industrial residues of local production and they have the potential to be used as prebiotics, and could be used in food related applications.

Keywords: Agro industrial residues, xylanase, xylooligosaccharides, enzymatic hydrolysis, prebiotics.

LISTA DE FIGURAS

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

CAPÍTULO IV

LISTA DE TABELAS

CAPÍTULO II

CAPÍTULO III

SUMÁRIO

INTRODUÇÃO

Os materiais lignocelulósicos compreendem resíduos provenientes de agroindústrias, florestas, tratamento de resíduos sólidos municipais, gramas perenes, entre outros (Balat, 2011). Os maiores componentes da parede celular destes materiais são a celulose, a hemicelulose e a lignina, além de extrativos e compostos inorgânicos (Kumar *et al.*, 2008), sendo que a proporção destes componentes varia na composição dependendo da espécie da planta, idade, tempo de colheita e condição ou estágio de crescimento. O grande acúmulo de resíduos lignocelulósicos provém em grande parte do processamento de culturas agrícolas (Cardona *et al.*, 2010; Rabelo *et al.*, 2009).

No Brasil, o estado do Rio Grande do Sul é o maior produtor de arroz e o terceiro maior produtor de soja, e consequentemente de resíduos provenientes do processamento desses grãos. Na safra 2016/2017 cerca de 12 milhões de toneladas de casca de arroz foram produzida no Brasil e a produção de soja no Brasil atingiu aproximadamente 113 milhões de toneladas em 33 milhões de hectares de área plantada, sendo que 16,5 % da produção ocorreu no Rio Grande do Sul (Conab, 2017). A casca do grão de soja representa o maior subproduto das indústrias processadoras dessa semente e constitui por volta de 8 % de todo o grão (Restle *et al.*, 2004).

Além disso, no Brasil, a malteação é o principal uso econômico da cevada, e o país produz 30 % da demanda da indústria cervejeira (Embrapa, 2012). A produção brasileira de cevada está concentrada na Região Sul, em especial no Rio Grande do Sul, que contribui com 70 % da produção nacional do cereal. A cevada é cultivada em mais de 140 mil hectares e a produção é de aproximadamente 420 mil toneladas (Agronline, 2012). Malte é o produto resultante da germinação e posterior dessecação do grão de cevada (*Hordeum sativum*) ou de outros cereais, é um material lignocelulósico, contendo cerca de 17 % de celulose, 28 % de polissacarídeos não-celulósicos, principalmente arabinoxilanos e 28 % de lignina (Mussato *et al.*, 2006). A composição rica em hemicelulose desses substratos demonstra potencial em bioprocessos para a produção de diferentes metabólitos e compostos como enzimas e prebióticos (Iqbal *et al.*, 2013).

Os fungos filamentosos realizam a degradação enzimática da porção lignocelulósica dos substratos pela elaboração das enzimas, celulases e xilanases, que são envolvidas na degradação de ligninoceluloses (Qinnghe *et al.*, 2004). Estas enzimas hidrolíticas são cruciais para a bioconversão da fração de celulose e hemicelulose em

açúcares simples para posterior fermentação e formação de produtos com alto valor agregado (Peplow, 2014).

O custo e o baixo rendimento dessas enzimas são os maiores problemas para aplicação industrial (Kanj *et al*., 2004). No entanto, investigações vem sendo realizadas quanto à habilidade de linhagens de diferentes microrganismos hidrolisarem celulose e hemicelulose utilizando substratos disponíveis e a preços acessíveis. Muitos trabalhos estão sendo direcionados ao desenvolvimento de uma alta produção microbiana enquanto focam o aprimoramento do processo de cultivo (Bortolazzo, 2011).

Os xilooligossacarídeos (XOS) são produzidos a partir de materiais lignocelulósicos (LCMS) contendo xilanos por métodos químicos, hidrólise enzimática direta de um substrato susceptível ou uma combinação de tratamentos químicos e enzimáticos (Aachary e Prapulla, 2011). A purificação do XOS obtido por transformação enzimática de substratos contendo xilanos é facilitada pelo tratamento químico prévio da matéria-prima lignocelulósica, bem como pela ação específica de xilanases (Moure *et al*., 2006).

Xilooligossacarideos são oligômeros formados por unidades de xilose, cujas características vêm conquistando espaço crescente na indústria farmacêutica, química fina e como ingrediente alimentar (Manrich, 2012). São considerados prebióticos, uma vez que promovem seletivamente o crescimento de probióticos como *Lactobacillus* e *Bifidobacterium*, promovendo uma série de benefícios à saúde humana, como a redução da constipação intestinal, a promoção da digestão e a absorção de nutrientes, a prevenção de infecções gastrintestinais e a inibição do crescimento de microrganismos patogênicos (Menezes e Durrant, 2008).

OBJETIVOS

Objetivo Geral

O objetivo geral do presente trabalho é a utilização de resíduos lignocelulósicos prontamente disponíveis no Rio Grande do Sul, advindos da agroindústria, para a produção de xilooligossacarídeos por hidrólise enzimática proveniente de cultivos de fungos filamentosos, e seu posterior uso como substrato para propagação de bactérias ácido-láticas para testar, preliminarmente, o potencial dos XOS obtidos como prebióticos.

Objetivos Específicos

- Seleção de fungos filamentosos para a produção de xilanase com a utilização de casca de soja, casca de arroz e bagaço de malte;
- Produção da enzima xilanase através de cultivo submerso e cultivo em estado sólido;
- Análise secundária da produção de enzimas celulolíticas e hemicelulolíticas, como celulase, β-glicosidase, β-xilosidase.
- Otimização do processo de produção de xilanase;
- Produção do xilooligossacarídeo por método enzimático utilizando-se as xilanases obtidas nos cultivos fungicos;
- Avaliação do potencial prebiótico dos XOS em culturas de bactérias ácido-láticas.

CAPÍTULO I: REVISÃO BIBLIOGRÁFICA

1.1 Materiais Lignocelulósicos

Com a incessante demanda das atividades agrícolas cresce o acúmulo destes resíduos gerando a deterioração deste ambiente e perda de recursos, com contribuição significante para o problema da reciclagem e conservação da biomassa. Diversos processos são empregados para utilização desses materiais, transformando-os em compostos químicos e produtos com alto valor agregado, tais como o álcool, enzimas, ácidos orgânicos, aminoácidos, entre outros. A utilização de resíduos lignocelulósicos em bioprocessos é uma racional alternativa para a produção de substratos e uma ajuda para solucionar problemas de poluição ambiental (Pandey *et al.*, 2000).

Os resíduos agroindustriais são constituídos por três principais frações poliméricas, celulose, hemicelulose e lignina, em uma estrutura cristalina vegetal altamente estável que deve ser rompida mediante pré-tratamentos para disponibilizar a fração sacarídica (celulose e hemicelulose) na forma de açúcares fermentescíveis (Dias *et al.*, 2013). A composição química de resíduos lignocelulósicos é interessante ao ponto de vista biotecnológico, pois geralmente esses resíduos são compostos de 20 % a 60 % de celulose, 20 % a 30 % de hemicelulose e 15 % a 30 % de lignina (Behera e Ray, 2016).

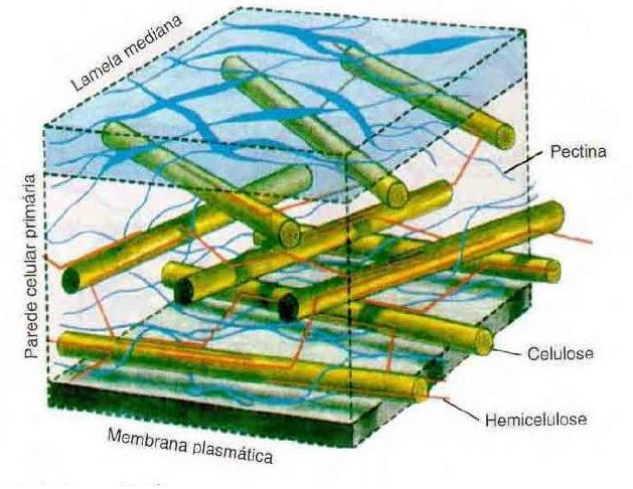

Figura 1. Modelo do complexo lignocelulolítico da parede celular (Raven *et al.*, 2001).

O principal componente dos materiais lignocelulósicos é a celulose, polissacarídeo formado por moléculas de glicose unidas através de ligações β-1,4-glicosídicas. Cadeias de celulose são estabilizadas e ligadas entre si por pontes de hidrogênio e essas fibras celulósicas são, por sua vez, recobertas por hemicelulose (polissacarídeo amorfo, composto por vários açúcares de cinco e seis carbonos, como arabinose, galactose, glicose, manose e xilose) e lignina (polímero aromático formado por três estruturas básicas fenólicas, o álcool p-cumarílico, álcool coniferílico e o álcool sinapílico) (Wyman *et al.*, 2005).

Muitos esforços vêm se empregando na indústria e na pesquisa para o aproveitamento da celulose, porém pouca atenção tem sido dada à hemicelulose, presente em uma fração de aproximadamente 30 % nos materiais lignocelulósicos (Dumon *et al.*, 2012). O fracionamento do principal componente da hemicelulose, xilana, pode ocorrer pela ação da enzima xilanase (Dhiman *et al.*, 2008). A figura 2A apresenta a estrutura de polímero de xilana, mostrando as ações das enzimas xilanolíticas e na figura 2B é mostrada a hidrólise da xilobiose por β-xilosidase. A cadeia principal de xilana é composta de ligações de 1,4- β-D-xilanopironosil que podem ser substituídas em vários graus com α-arabinofuranose; ácido glicurônico e ainda apresentam pequenas quantidades de ácido ferúlico e ácido p-cumárico (Li *et al.*, 2000).

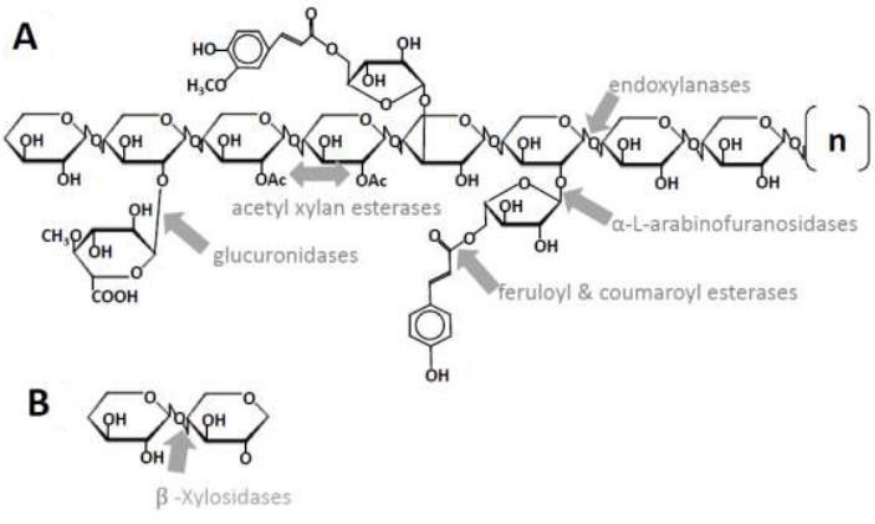

Figura 2. Estrutura A) de xilana, mostrando as ações das enzimas xilanolíticas; B) da xilobiose pela ação de β-xilosidase. (Kirikyali e Connerton, 2015)

As biomassas lignocelulósicas vêm se destacando ao longo do tempo e tornando-se cada vez mais alternativa promissora, devido à sua grande quantidade de material passível à fermentação (Rabelo *et al.*, 2014).

1.1.1 Casca de Arroz

O arroz (*Oryza sativa*) é um dos cereais mais produzidos e consumidos no mundo, caracterizando-se como principal alimento para mais da metade da população mundial. Sua importância é destacada principalmente em países em desenvolvimento, tais como o Brasil, desempenhando papel estratégico em níveis econômico e social (Walter *et al.*, 2008).

O Brasil é considerado um dos maiores produtores de arroz, cerca de 12 milhões de toneladas segundo os dados do acompanhamento de safra da Companhia Nacional do Abastecimento para a safra de 2016/2017 (Conab, 2017). Para cada tonelada de grãos de arroz colhidos são gerados, em média, 220 kg de casca (EMBRAPA, 2012).

Após o processo de beneficiamento, a casca de arroz torna-se um rejeito agrícola, caracterizado como baixa densidade, elevado conteúdo de sílica, grande resistência à degradação e difícil digestibilidade (Saha e Cotta, 2008). A maioria das indústrias de beneficiamento de arroz impacta o meio ambiente por causa da queima da casca do arroz, da produção de fumaça, do escoamento de efluente nos valos ou rios, e a geração de poeira. Com isso, são necessários ações para conservar o meio ambiente e diminuir os impactos ecológicos, sendo uma das alternativas a utilização das cascas de arroz em outros processamentos (Ursini e Bruno, 2012).

A casca de arroz é um dos resíduos lignocelulósicos que atrai a atenção dos pesquisadores devido ao seu conteúdo de celulose (30-38%) e hemicelulose (10-12%) e ao seu potencial para produção de bioprodutos e biocombustíveis (Ang *et al.*, 2012). Alguns estudos já utilizaram a casca de arroz, como substrato, para produção de enzimas em cultivo em estado sólido, Masutti *et al.* (2012) e Bansal *et al.* (2012) produziram a enzima celulase, Singh *et al.* (2011) e Potumarthi *et al.* (2013), produziram xilanase e celulase, Zimbardi *et al.* (2013), produziram β-glicosidase, β-xilosidase e xilanase.

1.1.2 Casca de Soja

A casca de soja, um subproduto obtido da industrialização da soja, é a película que reveste o grão, e consiste de uma fonte rica em fibra de baixa lignificação, com um teor de proteína bruta de cerca de 12 % e um teor de fibra bruta ao redor de 36 %, tem grande destaque no cenário nacional, em virtude da grande produção brasileira de soja (Restle *et al.*, 2004).

A cada tonelada de soja processada, cerca de 2 % é transformada no resíduo casca de soja, gerada na etapa de limpeza do grão no processamento da soja. Este resíduo é o de maior valor comercial em uma indústria processadora de soja, sendo que a sua principal utilização é como ingrediente na alimentação animal. Outra aplicação é a adição no farelo de soja com alto teor de proteína, desta forma o farelo passa a ter um teor de proteína adequado agregando valor a resíduos (Pukasiewicz *et al.*, 2004).

O décimo segundo levantamento da safra brasileira de soja na temporada 2015/16, realizado pela Conab, apresentou 33.251,9 mil hectares de área plantada para a soja, correspondendo a 95.434,6 mil toneladas, sendo 28.797,4 mil hectares para a região centro-sul (86.508,6 mil toneladas) e 4.454,5 mil hectares para o norte-nordeste (8.926,0 mil toneladas) (CONAB, 2016).

Asha e Prema (2006) e Park *et al.* (2002) em seus estudos sugerem a casca de soja como um resíduo agroindustrial com potencial para processos de produção de xilanase. Em virtude do seu valor nutritivo, a casca de soja pode ser utilizada na alimentação humana como ingrediente para elaboração de pães, biscoitos, bolos, *cookies* e *snacks*, principalmente em produtos à base de cereais no qual ocorre a complementação mútua de aminoácidos essenciais, além de aumentar a ingestão de fibras alimentares por dose diária (Oliveira, 2007).

A casca de soja é um material lignocelulósico, composto basicamente de açúcares fermentáveis polimerizados como celulose (glicose) e hemicelulose (xilose e arabinose) (Jeffries e Jin, 2000). Portanto, a sua hidrólise para liberar açúcares fermentáveis é potencialmente interessante para bioconversão em bioetanol e outros produtos (Schimer-Michel *et al.*, 2008).

1.1.3 Bagaço de Malte

O mercado cervejeiro brasileiro é o terceiro maior do mundo, atrás apenas dos EUA e China, e em 2016 atingiu uma produção de 14,1 bilhões de litros por ano, segundo a CervBrasil (Portal Brasil, 2017). O setor produtor de cerveja tem se expandido e, consequentemente, produzindo mais resíduos, dentre os quais, o que tem maior impacto em produção e volume é o bagaço de malte (FAO, 2013)

Este provém do processo de obtenção do mosto, pela fervura do malte moído e dos adjuntos, que após a filtração, resultam num resíduo que é destinado para ração animal (Aquarone, 2001). O bagaço de malte é gerado em quantidades de 14-20 kg a cada 100 litros de cerveja produzida (Cordeiro *et al*, 2012).

A composição química do bagaço de malte pode variar de acordo com a variedade e época de colheita da cevada, condições de moagem do malte e tipo de adjuntos (milho, arroz, trigo e sorgo) incorporados no processo cervejeiro (Santos *et al*., 2003).

O bagaço de malte é constituído basicamente pelas cascas da cevada malteada, e se encontra disponível o ano todo, em grandes quantidades e a um baixo custo. Rico em fibras e proteínas é um material lignocelulósico contendo aproximadamente 17 % de celulose, 28 % de hemicelulose e 28 % de lignina (Mussato *et al*., 2006).

Alguns trabalhos tem avaliado a possibilidade de emprego do bagaço de malte em processos biotecnológicos como substrato para cultivo de microrganismos e/ou para produção de enzimas. Wang *et al*. (2001), encontraram uma boa eficiência biológica do fungo *Pleurotus ostreatus* quando cultivado em bagaço de malte e consideram este material como um dos melhores substratos para cultivo dessa espécie de fungo.

1.2 Fungos filamentosos

Os fungos pertencem ao Reino Fungi e são organismos heterotróficos, constituídos, na maioria, por sistemas de hifas, que obtêm nutrientes por absorção (Crous *et al*, 2009). Estes organismos encontram-se distribuídos ubiquamente na natureza e estão presentes no solo, no ar, em matéria orgânica e também em ambientes aquáticos (Hageskal *et al*., 2009).

Na indústria, aplicações de microrganismos resultam rendimentos econômicos consideravelmente altos, exemplos clássicos de processos microbiológicos são as

fermentações de bebidas alcoólicas, combustíveis, laticínios, ácidos, antibióticos (Soares *et al.*, 2010). Entre o grande número de microrganismos capazes de produzir enzimas úteis, fungos filamentosos são particularmente interessantes, devido à alta produção de enzimas endo e extracelulares de grande potencial industrial (Guimarães *et al.*, 2006).

Os fungos filamentosos evoluíram para secretar relativamente grandes quantidades de enzimas que degradam os carboidratos dos materiais lignocelulósicos em açúcares fermentáveis. Neste caso, os fungos têm a capacidade de degradar a xilana, como o principal componente da hemicelulose que compõe a parede celular do resíduo, e desenvolver baterias de enzimas xilanolíticas que despolimerizam a xilana em ramos de carboidratos variáveis (Kirikyali e Connerton, 2015).

No entanto, nem todos os fungos são capazes de sintetizar enzimas lignolíticas, esta atividade especializada é reservada em grande parte aos gêneros *Armillaria mellea*, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Echinodontium taxodii*, *Aspergillus* sp. e *Fusarium* sp., que têm a capacidade de produzir tais enzimas (Shi *et al.*, 2014).

1.3 Enzimas hemicelulolíticas e celulolíticas

A produção de enzimas é uma área da biotecnologia em expansão e vem sendo amplamente estudada, movimentando bilhões de dólares anualmente (Gonzales *et al.*, 2003). O desafio da biotecnologia é gerar enzimas capazes de degradar a lignocelulose, com o objetivo de desenvolver processos por via biológica, com linhagens capazes de metabolizar as hexoses e pentoses provenientes dos materiais lignocelulósicos (Octave e Thomas, 2009).

A etapa de hidrólise consiste na degradação das cadeias poliméricas da celulose em monômeros de glicose e de hemicelulose em monômeros de xilose e/ou arabinose. Na hidrólise enzimática são utilizadas enzimas, que estas podem ser comerciais, mas devido ao alto custo podem ser secretadas por microrganismos (Carvalho, 2011). A produção e o uso de enzimas de origem microbiana, sob uma forma controlada constituem uma razoável parte do setor da indústria biotecnológica (Bortolazzo, 2011).

As celulases são enzimas capazes de hidrolisar materiais lignocelulósicos, liberando açúcares fermentescíveis, como a glicose (Castro e Pereira, 2010). O

complexo enzimático de celulases é composto principalmente por endoglucanases, exoglicanases e ß-glicosidases (Carvalho, 2011).

Além da celulase, vem sendo estudado a adição das enzimas hemicelulolíticas ao meio racional, como a xilanase, a fim de promover uma melhor eficiência da hidrólise. A xilanase teria a função de atuar sob a hemicelulose e converter esses compostos em xilose (Maeda *et al.*, 2011).

1.3.1 Xilanases

Xilanase, como exemplo a β-1,4-endoxilanase (EC 3.2.1.8), é a principal enzima responsável pela clivagem das ligações na cadeia principal de xilana, componente presente nos hidrolisados de materiais lignocelulósicos (Belfaquih *et al.*, 2002).

Para produção de XOS por enzimas, são necessários complexos enzimáticos contendo enzimas β-1,4-endoxilanases e/ou β-xilosidases, ambos podendo reduzir a xilana à xilose. Estas enzimas podem ser diretamente adicionadas para a reação, imobilizadas ou produzidas por microrganismos (Yoon *et al.*, 2006).

Diversos estudos mostram uma grande variedade de bactérias, leveduras e fungos filamentosos que produzem xilanase. A partir de uma perspectiva industrial, os fungos são interessantes devido a liberação extracelular de xilanases ser mais elevada (Sridevi e Charya, 2011). Entre os fungos filamentosos, os gêneros *Aspergillus* e *Trichoderma* são destaque na produção de xilanase (Polizeli *et al.*, 2005).

Essas enzimas catalisam a quebra das ligações glicosídicas do esqueleto da xilana (Figura 3), resultando na diminuição do grau de polimerização deste substrato e formação de XOS. De modo geral, as xilanases são glicoproteínas monoméricas, com massas moleculares a partir de 20 kDa (Patel e Savanth, 2015).

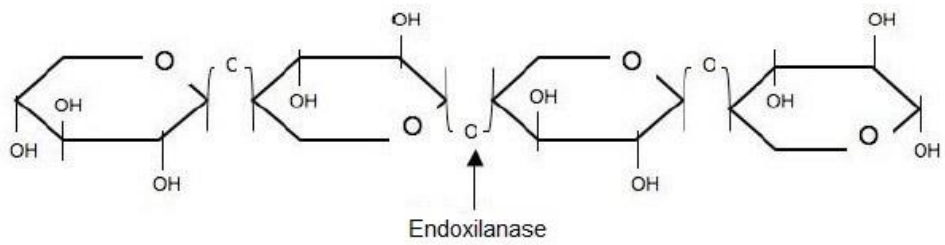

Figura 3. Ponto de clivagem da xilana por β-1,4-endoxilanase (Adaptado por Goldman, 2009).

As enzimas xilanases possuem grande potencial para aplicações industriais, principalmente para a bioconversão de materiais lignocelulósicos em açúcar e em outras substâncias úteis, e são utilizadas na clarificação de sucos e vinhos, além de no melhoramento da qualidade nutricional em panificação (Khurana *et al*., 2007).

β-xilosidases, em sua maioria, estão associadas a leveduras e bactérias, mas encontram-se em meio de culturas de alguns fungos filamentosos (Saha e Bothast, 1999). São muito utilizadas para hidrolisar os oligossacarídeos em açúcares simples (Saha, 2003).

As β-xilosidases (EC 3.2.1.37) são capazes de hidrolisar xilobiose e xilooligosacarídeos de cadeia curta (Knob *et al*., 2010). A xilosidase é responsável pela remoção da causa da inibição das endoxilanases, uma vez que hidrolisa xilobiose, aumentando assim a eficiência de hidrólise da xilana (Collins *et al*., 2005; Polizeli *et al*., 2005)

A adição de enzimas, como as β-xilosidases, podem melhorar o valor nutritivo dos alimentos (Omogbenigun *et al*., 2004). Na indústria de alimentos, as β -xilosidases são utilizadas na extração e clarificação de sucos, com vantagens na produção, operação e qualidade do produto final (Polizeli *et al*., 2005).

1.3.2 Celulases

As celulases (EC 3.2.1.4) são enzimas que constituem um complexo capaz de atuar sobre materiais celulósicos, promovendo sua hidrólise. Estas enzimas são biocatalisadores altamente específicos que atuam em sinergia para a liberação de açúcares, dos quais glicose é o que desperta maior interesse industrial. A classificação das celulases, de acordo com seu local de atuação no substrato celulósico, as dividem em três grandes grupos: endoglucanases (EnG), que clivam ligações internas da fibra celulósica; exoglucanases (ExG), que atuam na região externa da celulose; e β-glicosidases (BG), que hidrolisam oligossacarídeos solúveis em glicose (Lynd *et al*., 2002).

O sistema da celulase de fungos foi interpretado em termos de desenvolvimento substancial biológico molecular e bioquímico para o fungo *Tricoderma reesei*, o

primeiro fungo a ser utilizado na produção industrial de celulase, permanecendo ainda como a fonte mais utilizada (Wyman, 1999).

Celulases podem ser produzidas a partir de resíduos lignocelulósicos, pois estes apresentam-se como um substrato rico em celulose para a produção da enzima (Vinzant *et al.*, 1997).

β-glicosidases (β-D-glucoside glucohidrolases; EC3.2.1.21) catalisam a hidrólise de β-glicosideos. Muitas delas demostram também atividade sintética via hidrólise inversa ou transglicosilação. β - glicosidases têm um grande potencial para serem utilizadas em vários processos biotecnológicos, como sacarificação de bagaço de cana, liberação de compostos aromáticos na fabricação de vinho (terpenos), possuem capacidade antioxidante quando liberam compostos fenólicos (Bhatia *et al.*, 2002; Daroit *et al.*, 2007). β – Glicosidases, junto com pectinases, podem auxiliar na liberação de aromas e características voláteis de frutas e vegetais, o que é de grande importância para indústrias de alimentos (Bhat, 2000).

A capacidade de β- glicosidase em hidrolisar diferentes substratos glicosídicos a torna apropriada para vários processos industriais, incluindo a hidrólise enzimática de celulose, a fim de obter açúcares fermentescíveis e a produção de alimentos funcionais derivados de lignocelulósicos. Essa enzima também é utilizada na indústria de sucos e bebidas, onde pode melhorar a qualidade aromática de vinhos e outros derivados da uva (Leite *et al.*, 2008).

β–glicosidases de boa funcionalidade são capazes de serem produzidas por fungos, como *Penicillium viridicatum*, *Trichoderma reesei*, *Pycnoporus sanguines*, e estas são utilizáveis em vários processos biotecnológicos, por serem enzimas com atividade elevada na hidrólise (Smaali *et al.*, 2007). A sua aplicação na conversão de biomassa constituida de celulose a açúcares fermentecíveis para a produção de diversos produtos, é uma área intensivamente estudada (Chen *et al.*, 2007).

1.3.3 Enzimas recombinantes

Com o intuito de incrementar os níveis de produção de enzimas, diferentes técnicas podem ser utilizadas (Castro e Pereira, 2010). A expressão heteróloga é a principal ferramenta para a produção de xilanases a nível industrial. A engenharia genética facilita a expressão em larga escala já que as aplicações industriais exigem

enzimas mais baratas, com elevados níveis de expressão e secreção eficiente (Ahmed *et al.*, 2009).

A espécie que apresenta potencial para expressão heteróloga de proteínas é o *A. nidulans*. Esse microrganismo já foi utilizado como hospedeiro para produção de enzimas como a xilanase. Muller *et al.* (2015) expressaram o gene *xynB* de *Penicillium funiculosum* em *A. nidulans*, o que demonstrou produção de xilanase.

Para a construção do fungo utilizado neste trabalho, Segato *et al.* (2012) utilizaram uma xilanase codificada pelo gene *xynC* de *Penicillium funiculosum* para expressão no fungo *A. nidulans*. O vetor de expressão pEXPYR (Figura 4) foi utilizado para a superexpressão heteróloga da enzima xilanase. O vetor contém um promotor induzível por maltose, uma sequência de peptídeo sinal para secreção, duas marcas de seleção que permitem seleção positiva e negativa para resistência a zeomicina e um gene de marca auxotrófica *pyrG* de *Aspergillus niger* (Segato *et al.*, 2012).

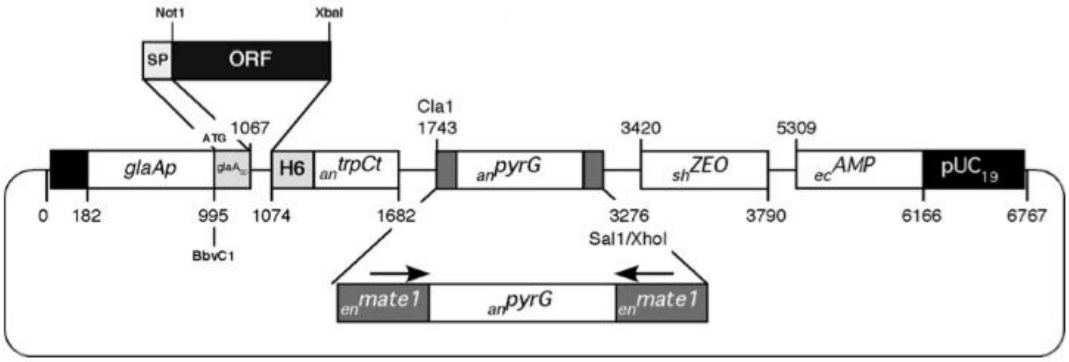

Figura 4. Vetor pEXPYR para recombinação de *A. nidulans*. (Segato *et al.*, 2012).

1.4 Cultivos microbianos em estado estado sólido e submerso

Em bioprocessos, o foco é a utilização de microrganismos para obtenção de produtos biotecnológicos. Processos biotecnológicos têm oferecido grandes possibilidades de inovações, como o desenvolvimento e melhoria de alimentos para humanos e animais, a produção e a utilização de novas enzimas, desenvolvimento de insumos e produtos farmacêuticos, dentre outras subáreas relacionadas (Pereira *et al.*, 2008). Bioaromas e oligossacarídeos, por exemplo, são duas classes de substâncias que podem ser produzidas biotecnologicamente por meio de bioprocessos microbiológicos (Bicas *et al.*, 2010).

Esses bioprocessos podem ocorrer em cultivo em estado sólido (CES), que é definido como o cultivo de microrganismos em substratos sólidos insolúveis na ausência (ou quase) de água livre. Nesse caso, o substrato fornece umidade suficiente para o desenvolvimento do microrganismo e funcionamento de seu metabolismo. Uma das características desse tipo de cultivo é a fase sólida atuar como fonte de carbono, nitrogênio e demais componentes, além de servir como suporte para o crescimento microbiano. Por esse motivo é interessante que se utilizem substratos com composição rica em nutrientes essenciais e, que, ao mesmo tempo, apresentem boa porosidade, para que o ar necessário ao desenvolvimento microbiano possa chegar ao microrganismo (Pandey, 2003).

Em cultivo em estado submerso (CS), o meio é constituído basicamente por água e nutrientes nela dissolvidos. Os processos microbianos de produção enzimática ocorrem na maioria das vezes em escala industrial sob condições de cultivo submerso (Castro e Pereira, 2010). Por volta de 75 % das enzimas produzidas na indústria é proveniente de cultivo submerso devido a facilidade de obtenção destes produtos (Subramaniyam e Vimala, 2012). Porém, o CES em relação a outros métodos fermentativos, apresenta grande rendimento produtivo, pouca produção de resíduos, utilização de substratos de baixo custo, baixo requerimento de água e energia, baixa possibilidade de contaminação durante o cultivo e exige equipamentos mais simples (Pandey, 2003).

1.5. Probióticos e Prebióticos

O trato gastrintestinal humano é um micro-ecossistema que possibilita o desempenho normal das funções fisiológicas do hospedeiro, a menos que microrganismos prejudiciais e potencialmente patogênicos dominem. Manter um equilíbrio apropriado da microbiota pode ser assegurado por uma suplementação sistemática da dieta com probióticos e prebióticos (McCabe *et al*., 2015).

Os probióticos são microrganismos vivos, administrados em quantidades adequadas, que conferem benefícios à saúde do hospedeiro (Roberfroid *et al*., 2010). Bactérias pertencentes aos gêneros *Lactobacillus* e *Bifidobacterium*, são mais frequentemente empregadas como suplementos probióticos para alimentos, uma vez que elas têm sido isoladas de todas as porções do trato gastrintestinal do humano saudável (Bielecka *et al*., 2002).

Prebióticos afetam beneficamente o hospedeiro, por estimularem seletivamente a proliferação e/ou atividade de populações de bactérias desejáveis no cólon (Roberfroid *et al.*, 2010). Os prebióticos são carboidratos não-digeríveis, incluindo diversos oligossacarídeos que fornecem carboidratos que as bactérias benéficas do cólon são capazes de fermentar (Cummingns e Macfarlane, 2002)

1.5.1 Xilooligossacarídeos (XOS)

Os xilooligossacarídeos (XOS) são os oligômeros constituídos de unidades de xilose, exibem excelente efeito e benefícios à saúde como prebiótico quando consumidos como parte da dieta, apoiando o crescimento de *Bifidobactérias* e *Lactobacillus* (Falck *et al.*, 2013). Devido ao seu papel como prebióticos, estes são usados em alimentos, indústrias de rações e produtos farmacêuticos (Deutschmann e Dekker, 2012).

Deste modo, existe uma procura crescente para a produção de XOS, geralmente produzidos a partir de resíduos agroindustriais (farelo de trigo, bagasso de cana de açúcar, milho) ricos em xilana através de uma variedade de métodos, como por exemplo, tratamento térmico, ácido, alcalino ou enzimático. Os processos enzimáticos empregando xilanases oferecem vantagens devido às condições de reação suaves, melhores especificidades, rendimentos mais elevados e ausência de qualquer subproduto (Vázquez *et al.*, 2000).

A produção de XOS ocorre pela ação enzimática, utilizando a enzima xilanase que hidrolisa ligações β-1,4 glicosídicas em xilanos. Na natureza, sistemas de enzimas xilanolíticas consistem de endoxilanases (1,4-β-D-xilano xiloidrolases, EC 3.2.1.8), β-D-xilosidases (1,4 β-xilosil xilanoidrolases, EC 3.2.1.37) e enzimas de desramificação (esterases). Todas estas enzimas agem de forma sinergética para converter xilana em seus açúcares constituintes. O aumento de xilose no meio causa repressão catabólica, portanto, a diminuição da produção de endo e exoxilanases e a diminuição dos XOS pode se dar também pela hidrólise dos mesmos pelas β–xilosidases (Akpinar *et al.*, 2009).

INTRODUÇÃO AOS CAPÍTULOS II, III e IV

Os Capítulos II, III e IV estão apresentados na forma de artigos científicos. Em cada um destes capítulos consta a introdução, os materiais e as metodologias empregadas, juntamente com a discussão dos resultados obtidos e as conclusões pertinentes a cada etapa de trabalho desenvolvida.

O primeiro artigo (Capítulo II - "Screening of filamentous fungi to produce xylanase and xylooligosaccharides in submerged and solid-state cultivations on rice husk, soybean hull, and spent malt as substrates") foi conduzido com o objetivo de selecionar o fungo maior produtor de xilanase utilizando resíduos agroindustriais. Este artigo foi publicado ao periódico World Journal of Microbiology and Biotechnology.

O segundo artigo (Capítulo III – "Comparative production of xylanase and the liberation of xylooligosaccharides from lignocellulosic biomass by *Aspergillus brasiliensis* BLf1 and recombinant *Aspergillus nidulans* XynC A773") foi conduzido com o objetivo de produzir xilanase a partir de um fungo filamentoso e um fungo recombinante, ou seja, geneticamente modificado, utilizando resíduo lignocelulósico como substrato, definindo parâmetros de processo e a produção de xilooligossacarídeos. Este artigo foi aceito ao periódico International Journal of Food Science & Technology.

No terceiro artigo (Capítulo IV – "Xylooligosaccharides production by fungi cultivations in rice husk and their application as substrate for lactic acid bacteria growth") foi realizada a produção de xilanase para aplicação em substrato e produção de xilooligossacarídeo, além de avaliar o seu efeito prebiótico frente a microrganismos probióticos. Este artigo foi aceito ao periódico Bioresource Technology Reports.

CAPÍTULO II: SCREENING OF FILAMENTOUS FUNGI TO PRODUCE XYLANASE AND XYLOOLIGOSACCHARIDES IN SUBMERGED AND SOLID-STATE CULTIVATIONS ON RICE HUSK, SOYBEAN HULL, AND SPENT MALT AS SUBSTRATES

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Screening of filamentous fungi to produce xylanase and xylooligosaccharides in submerged and solid-state cultivations on rice husk, soybean hull, and spent malt as substrates

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Abstract

We investigated the enzymatic complex produced by selected fungi strains isolated from the environment using the agro-industrial residues rice husk, soybean hull, and spent malt as substrates. Microbial growth was carried out in solid-state cultivation (SSC) and in submerged cultivations (SC) and the enzymatic activities of xylanase, cellulase, β-xylosidase, and β-glucosidase were determined. All substrates were effective in inducing enzymatic activities, with one strain of *Aspergillus brasiliensis* BLf1 showing maximum activities for all enzymes, except for cellulases. Using this fungus, the enzymatic activities of xylanase, cellulase, and β-glucosidase were generally higher in SSC compared to SC, producing maxima activities of 120.5, 25.3 and 47.4 $U.g^{-1}$ of dry substrate, respectively. β-xylosidase activity of 28.1 $U.g^{-1}$ of dry substrate was highest in SC. Experimental design was carried out to optimize xylanase activity by *A. brasiliensis* BLf1 in SSC using rice husk as substrate, producing maximum xylanase activity 183.5 $U.g^{-1}$ dry substrate, and xylooligosaccharides were produced and characterized. These results suggest *A. brasiliensis* BLf1 can be used to produce important lytic enzymes to be applied in the preparation of xylooligosaccharides.

Keywords: Agro-residues; *Aspergillus brasiliensis* BLf1; Xylanase; Xylooligosaccharides; Solid-state cultivation; submerged cultivations.

Introduction

Lignocellulosic biomass materials include wastes from agro-industries, plants, municipal solid waste, among others, with the largest part of them being derived from agricultural crops processing (Balat 2011; Cardona et al. 2010; Rabelo et al. 2009). The major cell wall components of these materials are cellulose, hemicellulose, lignin, extractives, and inorganic compounds (Kumar et al. 2008). These materials are not only an environmental problem when discarded, but also a source of reusable feedstock that can be used in physico-chemical and biotechnological processes. Among the uses, lignocellulosic biomass has been employed in processes to obtain fine chemical compounds, alcohols, enzymes, organic acids, amino acids, and more, turning the use of lignocellulosic residues in bioprocess as an alternative to produce fermentation substrates, helping to solve environmental pollution problems (Pandey et al. 2000).

Rice and soybean are two of the most cultivated crops worldwide for a long time, producing large amounts of residues, such as straws, husks, and hulls coming from the industrial processing of these grains (Wang et al. 1997). Another economic important grain is barley for malt production, which produces a sugar-rich residue that could be useful as substrate in bioprocess (Farias et al. 2009).

The production of cellulolytic and xylanolytic enzymes under solid-state cultivation (SSC) and submerged cultivation (SC), using inexpensive and easily available agricultural residues as substrates, could contribute to the reduction of these costs (Sukumaran et al. 2009). The major problem for a broader application of enzymes in the process of biomass hydrolysis is their high cost.

The main enzymes involved in the general hydrolyses process of lignocellulosic materials are xylanase (1.4- β -D- xylan xylanohydrolase (EC 3.2.1.8)), cellulase (EC

3.2.1.4), β-xylosidase (EC 3.2.1.37), and β-glucosidase (EC 3.2.1.21). Xylanase is the most important enzyme to be used in the production of xylooligosaccharides (XOS), being involved in the cleavage of bonds in the chain of xylan components present in lignocellulosic materials producing xylose oligomers (Belfaquih et al. 2002). XOS have prebiotic potential and can be incorporated into many food products, seem to exert their nutritional benefits in various animal species, which have an intestinal tract populated by a complex, bacterial intestinal ecosystem. The production of XOS on an industrial scale is carried out from lignocellulosic materials rich in xylan by chemical and enzymatic methods and the latter is preferred by the food industry because of the by products (Aachary and Prapulla 2011). XOS may be present in food or can be added to industrial products (Nabarlatz et al. 2007). XOS with a degree of polymerisation (DP) between 2 and 5 are preferred in the production of functional foods because they are substrates for probiotic bacteria in the gastric tract of hosts (Kiran et al. 2013; Gullón et al. 2011).

Xylanases have other potential industrial applications, especially for the bioconversion of lignocellulose materials into sugars that are fermentable to ethanol and other useful chemicals. In the food industry, it is also used for juices and wines clarifications (Khurana et al. 2007).

Several studies have shown a wide variety of microorganisms from bacteria to filamentous fungi showing xylanase activities. Fungi, in particular, are interesting because they show extracellular enzymes and generally are reported to produce higher activities of xylanase than bacteria (Sridevi and Charya 2011). The choice of a fungal strain showing high expression and diverse cellulolytic and xylanolytic enzymes are important to obtain the enzymatic complex required to hydrolyse lignocellulosic biomass. In this context, Brazil has a huge microbial biodiversity in its many biomes,

which could be explored in the search for untested microorganisms having the special characteristics for this end (Delabona et al. 2012).

In the light of these considerations, the aims of this study were to screen different strains of fungi isolated from several environments over different agro-industrial residues as potential substrates to investigate their production of lytic enzymes, specially xylanase, which could be used for subsequent production of xylooligosaccharides, along with cellulase, β-xylosidase, and β-glucosidase because these enzymes are important in the processes of lignocellulosic biomass hydrolyses. Tests were carried out in both submerged and solid state cultivations using rice husk, soybean hulls and spent malt as substrates. Experimental design was employed to optimize the production of xylanase by the best strain and its potential to produce xylooligosaccharides was tested.

Materials and Methods

Agro-industrial residues.

The rice husk was provided by Extremo-Sul (Camaquã, RS, Brazil); the soybean hull was obtained from Solae (Esteio, RS, Brazil); the spent malt was provided by Ralf Bier Brewery (Alvorada, RS, Brazil). Rice husk and soybean hull were milled in a knife mill and all processed materials were stored in sealed plastic bags and kept at room temperature until experimentation. The spent malt was stored in sealed plastic bags and maintained refrigerated at 4 ^{o}C for further experiments, without any pre-treatments. These materials were the substrates to be used in the cultivations.

Substrates characterization.

To determine the major constituents of the residues, they were submitted to quantitative acid hydrolysis using 72 % (mass fraction) sulphuric acid solution, in a solid-liquid proportion of 1:10 (Dunning and Dallas 1949). Monosaccharides liberated by hydrolysis were determined by HPLC in order to estimate (after corrections for stoichiometry and sugar decomposition) the contents of cellulose (as glucan), hemicelluloses (as xylan and arabinan), and acetyl groups. The acid soluble lignin was determined by UV-spectrophotometry. Protein was determined as total nitrogen content by the Kjeldahl method, using the N × 6.25 as conversion factor and ash was determined by the AOAC (AOAC 2000). All determinations were carried out in triplicates.

Screening of fungi for xylanase production.

Screening of fungi was conducted considering xylanase activity because this is the most important enzyme for subsequent production of XOS. The screening was carried out in submerged cultivation using rice husk, soybean hull, and spent malt. Fungi, isolated by our group from several environments, were used in this work (Table 2). These fungi strains are part of BiotecLab laboratory cultures (ICTA, UFRGS, Brazil). The strain showing highest xylanase production was selected for further studies, using the different substrates, but the activities of cellulase, β-xylosidase and β-glucosidase were also evaluated because these enzymes are involved in the hydrolysis process of lignocellulosic materials.

The fungal isolates were inoculated in plates of potato-dextrose agar (PDA), and incubated at 30^0C during 96 h. The screening for submerged cultivation was performed in Erlenmeyer flasks filled up with 30 mL of basal medium (pH 5.5) containing 3% (mass fraction) of the tested substrates. The basal medium, has the following

composition (in $g.L^{-1}$) : 1.4 $(NH_4)_2SO_4$; 2.0 KH_2PO_4; 0.3 $MgSO_4.7H_2O$; 0.3 $CaCl_2$; 5.0 $mg.L^{-1}$ $FeSO_4.7H_2O$; 1.56 $mg.L^{-1}$ $MnSO_4.H_2O$; 2.0 $mg.L^{-1}$ $CoCl_2$; and 1.4 $mg.L^{-1}$ of $ZnSO_4.7H_2O$, pH 5.5. The Erlenmeyer flasks were sterilized by autoclaving at 121 °C for 15 min. After sterilization, the flasks were inoculated with one disk (0.5 cm in diameter) of $1x10^7$ spores (Qinnghe et al. 2004; Alves-Prado et al. 2010; Sridevi and Charya 2011), quantified by counting on the Neubauer chambers and incubated for 7 days at 30 °C, under agitation at 180 rpm. At the end of cultivation, the flask content was centrifuged at 4,500*g* at 4 °C for 15 min, and the supernatants were used as crude enzyme extracts for further analyses. All determinations were carried out in triplicates.

After the initial screening, the five fungi strains that produced highest activities of xylanase were selected to further investigate the production of this enzyme and the other lytic enzymes of interest for lignocellulosic materials by growing them in submerged and solid-state cultivations. In screening all determinations were carried out in duplicates.

The selected fungi strains had their DNA extracted to determine their genera and species.

Molecular characterization and phylogenitc analyses of the best xylanase fungi producers.

Filamentous fungi were cultured on Potato Dextrose Agar, incubated at 28 °C for 5 days, and genomic DNA extraction was performed according to commercial extraction kit GenomicWizard - Promega. ITS rRNA genes of the filamentous fungi were amplified from genomic DNA by PCR using the following sets of primers, respectively: ITS 1 (5′ TCCGTAGGTGAACCTGCGG-3’), ITS4 (5′

TCCTCCGCTTATTGATATGC-3'). The sequencing was carried out using the SANGER method, using Applied Biosystems, model 3730XL and sets of primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').

ITS rRNA sequences obtained from the isolates were assembled in a contig using the CodonCodeAligner V4.2.7 (LI-COR®). The identification of the microorganisms was achieved by comparing the contiguous rRNA gene sequences obtained with rRNA sequence data from reference and type strains available in the public database NCBI, RDP. The sequences were analyzed using the MEGA software version 6.0. The evolutionary distances were derived from the sequence-pair dissimilarities and calculated as implemented in MEGA. The phylogenetic reconstruction was done using the neighbor-joining method with Jukes-Cantor parameter, with bootstrap values calculated from 1.000 replicate runs, using the routines included in MEGA software.

Solid-state and submerged cultivations.

The solid-state cultivations of fungi were carried out using 250 mL Erlenmeyer flasks containing 5 g of substrate added of mineral salt medium composed of 6 $g.L^{-1}$ $NaNO_3$; 0.52 $g.L^{-1}$ KCl; 0.52 $g.L^{-1}$ $MgSO_4.7H_2O$; 1.52 $g.L^{-1}$ KH_2PO_4; 1 mL piridoxine solution (100 $mg.mL^{-1}$), 2 mL of trace elements, composed of ($g.L^{-1}$): 22 $ZnSO_4.7H_2O$; 11 H_3BO_3; 5 $MnCl_2.4H_2O$; 5 $FeSO_4.7H_2O$; 1.6 $CoCl_2.6H_2O$; 1.6 $CuSO_4.5H_2O$; 1.1 $(Mn_4)_6Mo_7O_{24}$; and 50 EDTA, pH 6.5, in order to achieve 60 % final moisture in the substrate. The flasks were sterilized by autoclaving at 121°C for 20 min, and were inoculated with one disk (0.5 cm in diameter) of $1x10^7$ spores, measured using the Neubauer chamber counting. The flasks were incubated for 10 days at 30 °C, with

samplings every other day. After cultivation, enzymatic extraction was performed using 40 mL of sodium acetate buffer 50 mM (pH 5.0). The suspensions were stirred at 180 rpm in an orbital shaker at 30 °C for 15 min. The solids were separated by centrifugation at 4,500 *g* at 4 °C for 15 min and the enzyme activity of the supernatant was assayed.

The submerged cultivations were carried out in Erlenmeyer flasks containing 30 mL of basal medium (pH 5.5) containing 3 % (mass fraction) of the tested substrates. The basal medium had the following composition ($g.L^{-1}$): 1.4 $(NH_4)_2SO_4$; 2.0 KH_2PO_4; 0.3g $MgSO_4.7H_2O$; 0.3 $CaCl_2$; 0.005 $FeSO_4.7H_2O$; 0.0016 $MnSO_4.H_2O$; 0.002 $CoCl_2$; and 0.0014 $ZnSO_4.7H_2O$, pH 5.5. The flasks were sterilized by autoclaving at 121 °C for 20 min. After sterilization, the flasks were inoculated with one disk (0.5 cm in diameter) of $1x10^7$ spores, measured as described above and incubated for 10 days at 30 °C, with sampling taking place every other day. The cultured extracellular fluid was centrifuged at 4,500 *g* at 4 °C for 15 min. The supernatants were used as crude enzyme extracts. Control submerged cultivations were performed using only the mineral medium without addition of the substrates to allow comparisons of the differences of enzymatic activities.

Enzymes assays: Xylanase and cellulase activities.

Xylanase and cellulase activities were assayed following similar procedures. In test tubes, 1 mL of enzymatic extracts obtained as described above, were incubated with 1 mL of 1 % solutions of either xylan (Sigma-Aldrich, USA) or carboxymethylcelullose solution (Sigma-Aldrich, USA) as substrates, in sodium acetate buffer (50mM, pH 5.0). The mixtures were left to react for 30 min at 50 °C. Reducing sugars liberated by hydrolysis of these substrates were quantified by the dinitrossalicylic method (Miller

1959). A unit of enzymatic activity was defined as the amount of enzyme producing 1 µmol of xylose or 1 µmol of glucose per min. Activities were expressed as $U.g^{-1}$ dry substrate. Results were expressed as the mean of duplicate measurements.

Enzymes assays: β -glucosidase and β-xylosidase activities.

The β–glucosidase (BG) and β–xylosidase (BX) activities were measured in a similar way, following the method described by Zheng and Shetty (2000), with some modifications. Both enzymes were assayed in a reaction mixture containing 100 µL sodium citrate buffer (50 mM, pH 4.8), 100 µL of 9 mM p-nitrophenyl-β-D-glucopyranoside (pNPG, Sigma-Aldrich, USA) for BG, or 100 µL 9 mM p-nitrophenyl-β-D-xylopyranoside (pNPX, Sigma-Aldrich, USA) for BX, and 100 µL of enzymatic extract. After incubation at 50 °C during 30 min, the reactions were stopped by adding 1.5 mL 500 mM sodium carbonate. The activities were estimated spectrophotometrically by reading the absorbance of the liberated p-nitrophenol at 400 nm. One unit of BG or BX were defined as the amount of enzyme required for the hydrolysis of 1 µmol of substrates per min, under the assay conditions. Specific activities were expressed as $U.g^{-1}$ dry substrate. Results were expressed as the mean of duplicate measurements.

Experimental design.

The fractional factorial experimental design $2^{(5-1)}$ was performed to obtain the best conditions for the activity of the xylanase enzyme using the best strain producer of xylanase as identified in the previous experiments. The variables and their coded and uncoded values are presented in Table 1, whereas in Table 6 are shown the 16 treatments obtained for the five variables, each at two levels. The design was constructed of sixteen factorial points and in each case, the xylanase activity was

determined after nine days of cultivation a 30 ^{0}. The production of xylooligosaccharides was also evaluated in these experiments in SSC.

Table 1. Tested variables and their different levels used in the fractional factorial experiment to determine the best conditions of *Aspergillus brasiliensis* BLf1 xylanase activity

Variables	Name	Coded levels	
		-1	+1
X_1	Particle size (mm)	1	2
X_2	Moisture (%)	60	80
X_3	pH	4.5	7.5
X_4	Inoculum size (spores.g^{-1})	10^5	10^8
X_5	Basal medium (mL)	0	4

Determination of xylooligosaccharides.

The cultivation extracts, prepared as described for the enzymatic extractions, were filtered through 0.22 µm membrane and the xylooligosaccharides were determined by high-performance liquid chromatography, using an Aminex HPX 42 column, Bio Rad, analysis conditions are: column temperature was 85 ^{o}C, using ultra-pure water as eluent with the flow 0.6 ml.min^{-1} and a refractive index detector. The concentration of oligosaccharides was quantitated using the peak areas compared to xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6). The sugar standards were obtained from Megazyme (Bray, Ireland).

Statistical analysis.

The analyses of results were carried out using Statistica 7.0 (Statsoft, USA). The significance of the regression coefficients was determined by Student's t-test applied to all determinations. All experiments were run in triplicates, unless otherwise mentioned. The results of the experimental design were analyzed using analysis of variance (ANOVA) at $p < 0.05$ and response surface analysis.

Results

Substrate characterization.

The compositions of the agro-industrial residues used as substrates are presented in Table 2. Rice husk has high amounts of ash (16.2 %) and lignin (25.5 %), but is low in hemicellulose content (11.2 %). Its cellulose content (37.4 %) is similar of that of soybean hull. Soybean hull, on the other hand, shows relatively low amounts of lignin (6.5 %) and ashes (0.6 %), and a high content of cellulose (38.3 %). Spent malt presents 17.9 % of cellulose and 21.8 % of hemicellulose, respectively.

Table 2. The composition of agro-industrial residues used in this research(1)

	Protein (%)	Ash (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Rice husk	1.9 ± 0.17^{c}	16.2 ± 0.08^{a}	37.4 ± 0.05^{a}	11.2 ± 0.01^{c}	25.5 ± 0.25^{a}
Soybean hull	8.5 ± 0.16^{b}	0.6 ± 0.25^{c}	38.3 ± 0.05^{a}	19.5 ± 0.03^{b}	6.5 ± 0.10^{c}
Spent Malt	12.8 ± 0.48^{a}	2.9 ± 0.11^{b}	17.9 ± 0.34^{b}	21.8 ± 0.01^{a}	19.9 ± 0.01^{b}

*Dry basis

(1) Same letters in the column do not differ significantly at 5 % probability by Tukey test.

Screening of fungi for xylanase activity.

The isolated strains tested in this work were grown on the three agro-residue substrates to check their xylanase activities and the results are shown in Table 3, with the highest activities being produced in spent malt cultures of the strains BLf6, BLf8, and BLf1.

Considering these results, the five fungi strains that presented the highest xylanase activities (BLf7, BLf6, BLf11, BLf1, and BLf8) were selected for further experiments to evaluate activities of other xylanolytic and cellulolytic enzymes in submerged and solid-state cultivation conditions.

Table 3. Xylanase activities of several fungi strains tested in this research in SC(#)

Fungi	Enzyme activity (U.g^{-1} dry substrate)*		
	Rice husk	Soybean hull	Spent malt
Pleorotus ostreatus[1]	4.44 ± 0.54^{c}	6.17 ± 1.66^{b}	24.33 ± 0.86^{a}
Ganoderma lucidon[1]	3.27 ± 0.18^{b}	9.98 ± 0.38^{a}	8.39 ± 0.26^{a}
Agrocybe aeregita[1]	1.25 ± 0.74^{b}	1.47 ± 1.02^{b}	8.82 ± 0.27^{a}
Lepista nuda[1]	1.02 ± 0.21^{b}	1.60 ± 0.25^{b}	6.74 ± 0.19^{a}
Lentinula edodes[2]	1.12 ± 0.17^{c}	2.31 ± 0.27^{b}	7.52 ± 0.22^{a}
BLf1	13.31 ± 1.20^{b}	8.85 ± 2.10^{b}	46.58 ± 1.70^{a}
BLf2	0.94 ± 1.34^{c}	6.08 ± 2.18^{b}	29.34 ± 1.04^{a}
BLf3	1.59 ± 2.16^{c}	8.29 ± 1.36^{b}	28.14 ± 1.00^{a}
BLf4	5.53 ± 2.01^{c}	7.91 ± 1.60^{b}	23.48 ± 0.82^{a}
BLf5	1.50 ± 2.21^{c}	7.87 ± 1.47^{b}	31.76 ± 1.13^{a}
BLf6	11.42 ± 2.22^{b}	7.47 ± 1.45^{c}	52.41 ± 1.91^{a}
BLf7	13.0 ± 0.58^{b}	8.19 ± 1.85^{c}	39.40 ± 1.42^{a}
BLf8	7.22 ± 0.92^{c}	12.64 ± 1.32^{b}	45.76 ± 1.66^{a}
BLf9	8.07 ± 0.16^{c}	13.07 ± 1.01^{b}	30.0 ± 1.07^{a}
BLf10	3.47 ± 0.26^{b}	11.10 ± 1.38^{b}	27.95 ± 0.99^{a}
BLf11	12.04 ± 0.25^{b}	9.60 ± 1.31^{b}	34.02 ± 1.22^{a}

(#), Same letters in the same line do not significantly differ at 5% probability by Tukey test. (*)Enzymatic activities measured at time of 5 days in SC. (1)Microorganisms isolated from decaying wood. (2)Microorganisms isolated from tissue culture of sporophore; BLf1 to BLf11, isolated from soil samples.

Fungi strains identification.

Fungal strains BLf1, BLf6, BLf7, BLf8, and BLf11 were identified by molecular methods. The analysis showed that isolates belong to the genera *Aspergillus* and *Penicillium*. In the phylogenetic tree (Figure 1), the strain BLf1 showed to be a strain of *Aspergillus brasiliensis.*

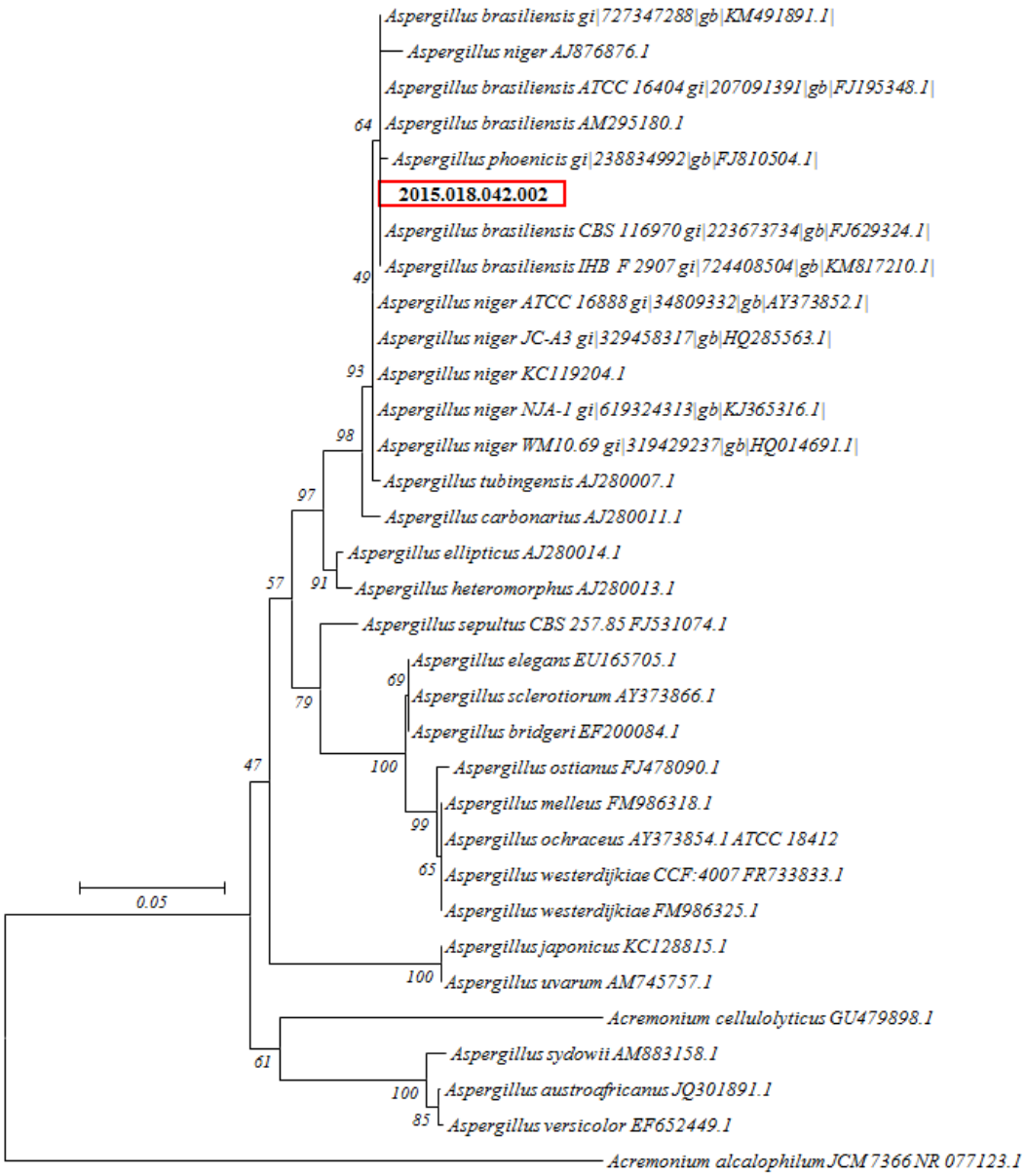

Fig 1. Phylogenetic analysis of partial ITS rDNA gene sequence of isolate BLf1 (2015.018.042.002) and related microorganisms. Built with the help of MEGA 6.0

software by the neighbor-joining method with Jukes-Cantor parameter and Bootstrap values (1000 replicate runs, shown as percent).

The isolates BLf6, BLf7, and BLf8 belong to the genus and species *Penicillium crustosum* (Figure 2), whereas BLf11 was classified as a new *Aspergillus* species (Figure 3).

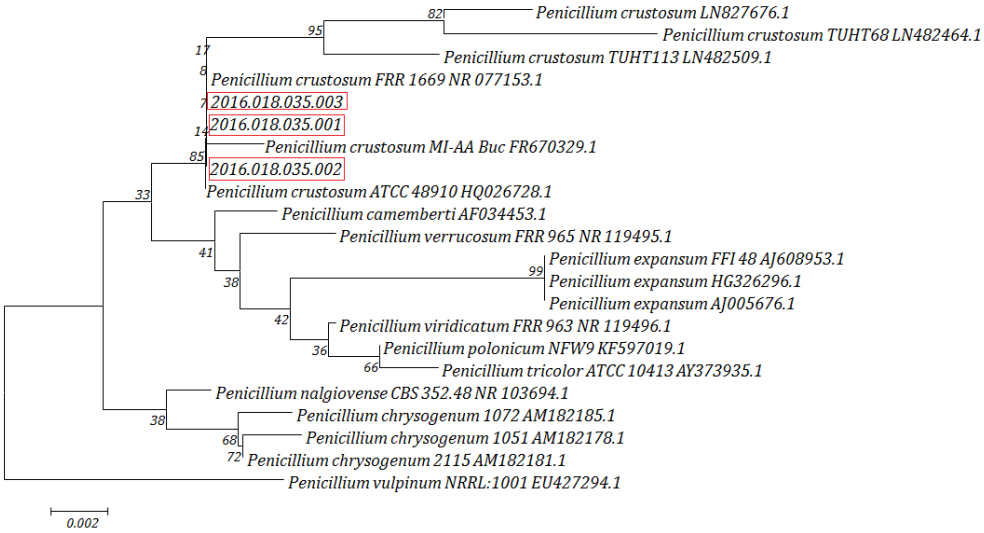

Fig 2. Phylogenetic analysis of partial ITS rDNA gene sequences of fungi isolates BLf6 (2016.018.035.003), BLf7 (2016.018.035.001), and BLf8 (2016.018.035.002), and related microorganisms. Built with the help of MEGA 6.0 software by the neighbor-joining method with Jukes - Cantor parameter and Bootstrap values (1000 replicate runs, shown as percent).

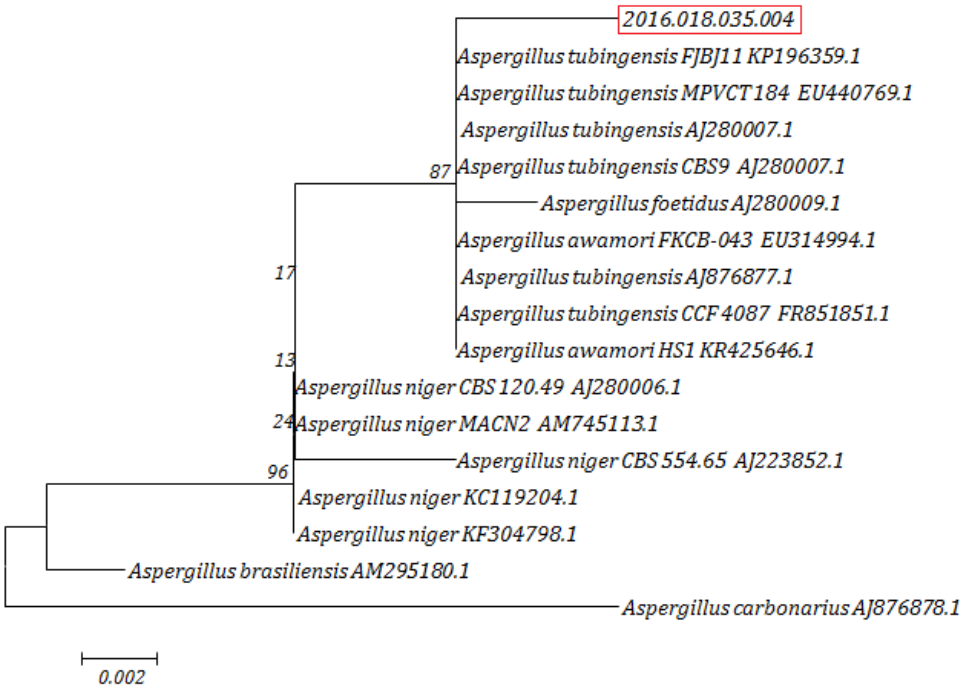

Fig 3. Phylogenetic analysis of partial ITS rDNA gene sequence of isolate BLf11 (2016.018.035.004) and related microorganisms. Built with the help of MEGA 6.0 software by the neighbor-joining method with Jukes-Cantor parameter and Bootstrap values (1000 replicate runs, shown as percent).

Enzymatic activities in solid-state cultivations of selected strains.

The five selected strains in the previous step were grown in solid-state and submerged cultivations to verify their potential to produce the lytic enzymes xylanase, cellulase, β-xylosidase, and β-glucosidase, enzymes that are important in the process of lignocellulosic biomass hydrolyses in general. In Table 4 are presented the results of enzyme activities of the five selected strains in SSC.

The strain of *A. brasiliensis* BLf1 was the microorganism showing the highest activities of xylanase (120.5), β-xylosidase (23.08), and β-glucosidase (47.77). Cellulase activity was highest (25.29) in cultures of *Aspergillus* sp. BLf11.

Enzymatic activities in submerged cultivations of selected strains.

The enzymatic activities of the screened fungi strains tested in SC and their controls are presented in Table 5. In this set of experiments, *A. brasiliensis* BLf1 was the strain producing highest activities of xylanase (113.85 $U.g^{-1}$ substrate), β-xylosidase (28.11 $U.g^{-1}$ substrate), and β-glucosidase (33.35 $U.g^{-1}$ substrate), using soybean hull and spent malt as inducers. The highest activity of xylanase in SC was obtained in cultures of *Aspergillus brasiliensis* BLf1 using spent malt as substrate. Cellulase activity was highest (20.69 $U.g^{-1}$ substrate) in cultures of *Penicillium crustosum* BLf6 in spent malt.

Experimental design.

Aspergillus brasiliensis BLf1 was the best xylanase producer using rice husk as substrate in SSC. Therefore, an optimization of this enzyme production was carried out using experimental design as a tool. The experimental design and the results of fractional factorial design 2^{5-1} for *A. brasiliensis* BLf1 are shown in Table 6. The enzyme activity varied from 27.4 to 183.5 $U.g^{-1}$ dry substrate.

Determination of xylooligosaccharides.

The highest concentrations of XOS produced by *A. brasiliensis* BLf1 growing on rice rusk in SSC were observed for extracts of treatment 11, and the chromatogram showing the XOS profiles is represented in Figure 4. The highest concentration of XOS was observed at 48 h of cultivation. The following XOS concentrations were obtained: 6.41 $mg.g^{-1}$ substrate of X2, 4.49 $mg.g^{-1}$ substrate of X4, 3.58 $mg.g^{-1}$ substrate of X5, and 48.4 $mg.g^{-1}$ substrate of xylose.

Table 4. Enzymatic activities of several fungi strains tested in SSC

		Enzyme activity (U.g^{-1} dry substrate)			
Fungi	Substrate	Xylanase	Cellulase	β-xylosidase	β –glucosidase
Penicillium crustosum BLf7	Rice husk	71.07 ± 0.02	2.47 ± 0.09	0.08 ± 0.02	0.62 ±0.02
	Soybean hull	38.12 ± 0.06	2.47± 0.03	0.73± 0.01	3.97± 0.05
	Spent Malt	69.9± 0.02	7.68 ± 0.02	4.33 ± 0.06	17.64 ± 0.08
Aspergillus sp. BLf11	Rice husk	117.98 ± 0.01	3.30 ±0.1	1.05 ± 0.01	7.80 ± 0.01
	Soybean hull	76.18 ± 0.09	**25.29 ± 0.01**	3.24 ± 0.01	9.48 ± 0.09
	Spent Malt	88.28 ± 0.04	18.94 ± 0.01	19.75 ±0.05	15.9 ± 0.14
Penicillium crustosum BLf8	Rice husk	101.51 ± 0.73	6.37 ± 0.01	0.43 ± 0.02	0.81 ± 0.01
	Soybean hull	28.01 ± 0.01	3.73 ± 0.02	2.51 ± 0.26	7.38 ± 0.01
	Spent Malt	98.5 ± 0.09	13.7 ± 0.01	4.25 ± 0.01	15.68 ± 0.03
Penicillium crustosum BLf6	Rice husk	19.29 ± 0.15	1.31 ± 0.02	0.02 ± 0.01	0.2 ± 0.01
	Soybean hull	4.09 ± 0.15	1.68 ± 0.02	0.09 ± 0.01	1.18 ± 0.09
	Spent Malt	16.26 ± 0.01	4.28 ± 0.22	0.2 ± 0.02	0.71± 0.03
Aspergillus brasiliensis BLf1	Rice husk	**120.5 ± 0.04**	1.23 ± 0.01	1.37 ± 0.01	2.64 ± 0.02
	Soybean hull	95.45 ± 0.13	5.01 ± 0.05	17.04 ± 0.03	26.4 ± 0.05
	Spent Malt	76.78 ± 0.08	7.18 ± 0.02	**23.08 ±0.18**	**47.77 ± 0.01**

Table 5. Enzymatic activities of several fungi strains tested in SC

			Enzyme activity (U.g^{-1} dry substrate)			
	Fungi	Substrate	Xylanase	Cellulase	β-xylosidase	β –glucosidase
SC	*Penicillium crustosum* BLf7	Rice husk	1.43± 0.01	0.41± 0.01	0.01± 0.01	0.08± 0.01
		Soybean hull	38.85± 0.03	0.94± 0.02	0.23± 0.02	3.05± 0.02
		Spent Malt	41.83 ± 0.01	4.31 ± 0.02	5.64 ± 0.08	11.08 ± 0.09
	Aspergillus sp. BLf11	Rice husk	57.9± 0.01	2.52± 0.09	2.06± 0.03	9.43± 0.05
		Soybean hull	59.61± 0.02	1.46± 0.11	11.63± 0.02	11.54± 0.01
		Spent Malt	63.31 ± 0.07	3.77 ± 0.25	15.33 ±0.01	11.85 ± 0.03
	Penicillium crustosum BLf8	Rice husk	51.6± 0.01	2.24± 0.02	0.23± 0.01	1.16± 0.01
		Soybean hull	34.8± 0.01	1.24± 0.05	4.42± 0.04	11.95 ± 0.02
		Spent Malt	64.03 ± 0.02	2.4 ±0.01	8.5 ± 0.02	11.75± 0.03
	Penicillium crustosum BLf6	Rice husk	4.53± 0.27	0.43± 0.01	0.17± 0.11	0.32± 0.03
		Soybean hull	5.17± 0.07	1.75± 0.07	0.95± 0.18	3.27± 0.02
		Spent Malt	61.98 ± 0.04	**20.69 ± 0.09**	2.89 ± 0.01	3.99 ± 0.18
	Aspergillus brasiliensis BLf1	Rice husk	90.67± 0.04	6.74± 0.01	2.94± 0.01	20.58± 0.1
		Soybean hull	70.51± 0.03	4.28± 0.02	**28.11 ±0.34**	29.29± 0.19
		Spent Malt	**113.85 ± 0.07**	6.79 ± 0.05	21.33± 0.51	**33.35 ± 0.01**
Control	*Penicillium crustosum* BLf7		0.29 ± 0.04	0.53 ±0.02	0.17 ± 0.01	1.72 ± 0.01
	Aspergillus sp. BLf11		0.48 ± 0.05	0.43 ±0.07	0.19 ± 0.02	1.80 ± 0.07
	Penicillium crustosum BLf8		0.52 ± 0.10	0.29 ±0.04	0.20 ± 0.05	1.53 ± 0.23
	Penicillium crustosum BLf6		0.60 ± 0.01	0.34 ±0.02	0.18 ± 0.02	2.08 ± 0.16
	Aspergillus brasiliensis BLf1		0.40 ± 0.04	0.44 ±0.04	0.18 ± 0.02	2.81 ± 0.14

Table 6. Experimental design and results of fractional factorial design 2^{5-1} to determine the best conditions of *Aspergillus brasiliensis* BLf1 xylanase activity

Treatment	Levels					Xylanase Activity ($U.g^{-1}$dry substrate)
	X_1	X_2	X_3	X_4	X_5	*A. brasiliensis* BLf1
1	-1	-1	-1	-1	1	139.1
2	1	-1	-1	-1	-1	84.5
3	-1	1	-1	-1	-1	153.0
4	1	1	-1	-1	1	155.3
5	-1	-1	1	-1	-1	77.5
6	1	-1	1	-1	1	127.1
7	-1	1	1	-1	1	115.9
8	1	1	1	-1	-1	27.4
9	-1	-1	-1	1	-1	132.3
10	1	-1	-1	1	1	150.3
11	-1	1	-1	1	1	183.5
12	1	1	-1	1	1	118.8
13	-1	-1	1	1	1	133.7
14	1	-1	1	1	-1	35.5
15	-1	1	1	1	-1	57.7
16	1	1	1	1	1	85.7

Discussion

The compositions of rice husk and soybean hull used in this work are similar to results reported by Saha et al. (2005), Hickert et al. (2013), and Schirmer-Michel et al. (2008), according to these authors, the cellulose amount varied from 30 to 38 %, hemicellulose varied from 10 to 12 %, and lignin from 3 % in the soybean hull and between 15 and 30 % in rice husk, respectively.

Spent malt has a general composition of 16 to 18 % of cellulose, 28 to 29 % of hemicellulose, and lignin around 27 to 28 % (Mussato et al. 2006). The high ash and lignin contents of rice husk are major problems concerning the bioconversion of this biomass. The lignin is associated to polyoses (hexoses and pentoses) through physical interactions and covalent bonds, fact that turns difficult its biodegradation, protecting the biomass against microbial action (Reading et al. 2003).

Soybean hull has a composition that is highly interesting in order to obtain readily fermentable glucose. On the other hand, spent malt shows a composition high in hemicellulose, which could provide xylan and arabinnan, the first polymer interesting in the production of XOS.

Spent malt contains a higher hemicellulose content than rice husk and soybean hull and its lignocellulosic matrix is also more easily available to the action of microorganisms enzymes because of its industrial processing, which includes thermal process and high protein content. These facts explain the highest xylanase activities obtained using this residue. On the other hand, the ash and lignin-rich rice husk, showed to be the poorest substrate concerning the enzyme activity.

Even at this preliminary screening stage, the results suggest high enzyme activities by some of these strains. For instance, Pandey and Pandey (2002), in their study to obtain cellulase-free thermostable fungal xylanases using different lignocellulosic biomass, obtained the maximal enzyme activities on oatmeal (55.2 $U.g^{-1}$dry weight substrate), followed by wheat, rice husk, bagasse and gram residue, showing that xylanase activities are directly related to the composition of the lignocellulosic substrate.

The strain BLf1, which showed the highest xylanase activity, was identified by conventional and molecular approaches. In the phylogenetic tree (Figure 1) the strain BLf1 was characterized as *Aspergillus brasiliensis,* which is described as morphologically being similar to other species of same genus, such as *A. niger*.

Phylogenetic analysis based on the ITS rRNA gene molecular marker showed that isolates BLf6, BLf7, and BLf8 belong to the genus and species *Penicillium crustosum*, supported by a high boostrap value (85 %), with this species (Figure 2). Finally, BLf11 showed a restricted level of sequence similarity observed with the species *Aspergillus tubingensis*, its closest match, showing 87 % of similarity, and this could be an indicative of a new *Aspergillus* species.

Surprisingly, the solid-state cultivation carried out with the five strains best producers of xylanase on rice husk, produced higher xylanase activities compared to the other substrates, whereas soybean hull was the best substrate for cellulase, and spent malt best for the other two enzymes. Clearly, results show that the differences in enzymatic activities are related to substrate composition and possibly their physical structures and the capacity to absorb water.

The genus *Aspergillus* is generally reported as the best microorganism to be cultured in solid-state fermentation because of the strong development of hyphae, allowing greater penetration in the substrate and the porous regions between particles and being less dependent on water activity (Souza et al. 2010).

The solid-state cultivation of *A. lentulus* on various low-cost agro-residues produced xylanase activities of 158.4 $U.g^{-1}$ of wheat bran, 153.0 $U.g^{-1}$ of corncob, 129.9 $U.g^{-1}$ of sugarcane bagasse, and 49.4 $U.g^{-1}$ of wheat straw (Kaushik et al. 2014). Park et al. (2002), reported xylanases activities in solid-state cultivations of *A. niger* on rice

straw, of 5 $U.g^{-1}$dry substrate after 9 days of growth at 28 °C. The results obtained in this work for the SSC of BLf1 are consistent with those of a good strain producer of this enzyme.

Concerning cellulase activities, the highest value was obtained in the cultures of *Aspergillus* sp. BLf11 in soybean hull. Bansal et al. (2012), studied the cellulase production by *A. niger* NS-2 on various agricultural and kitchen wastes and the authors reported cellulase activities between 1.0 and 310.6 $U.g^{-1}$ dry substrate using orange peelings and wheat bran as substrate, showing that cellulase activity was largely dependent on the fermentation carbon source. In this study, *Aspergillus* sp. BLf11 cellulase activities were comparable to reports on the literature for SSC using different substrates, suggesting that process optimization can further increase its production (Sales et al. 2010; Rodriguez-Zuniga et al. 2011).

The *Aspergillus brasiliensis* BLf1 showed the highest activities of of β-xylosidase, values compared to reports on the literature. Zimbardi et al. (2013) optimized culture conditions of *Colletotrichum graminicola* in wheat bran, reporting maximal β-xylosidase activities of 57.9 $U.g^{-1}$ dry substrate. *A. brasiliensis* BLf1 growing in spent malt also produced the highest activities of β-glucosidase. Delabona et al. (2013) studied the effect of initial moisture content in cultures of two Amazonian environment isolated strains of *A. niger*, using wheat bran as substrate, and obtained the highest activity of β-glucosidase of 105.8 $U.g^{-1}$ dry substrate. The activity of β-glucosidase is important for the efficient hydrolysis of cellulose. However, the highest activity of xylanase in the submerged cultivation was obtained in cultures of *Aspergillus brasiliensis* BLf1 using spent malt as substrate. As a general trend, rice husk was a poor substrate for enzyme activities in SC, probably because it shows high content of silica, making it less soluble in the medium.

Rani et al. (2014) optimized SC of *Thielaviopsis basicola* MTCC 1467 using rice straw as inducer to improve xylanase activities from 400 to 1,500 $U.g^{-1}$ dry substrate. Similar studies screening these enzymes in SC of various fungi produced activities that are reportedly compatible with results in this study. Gottschalk et al. (2013) observed highest β-xylosidase activities of 22.6 $U.g^{-1}$ substrate using wheat bran as carbon source and *A. awamori* 2B.361 U2/1. Anthony et al. (2003) reported cellulase activities lower than 20 $U.g^{-1}$ dry substrate (rice straw) in submerged cultures of *A. fumigatus* AR1. *Thermoascus aurantiacus miehe* was cultivated using wheat bran and sugarcane bagasse as inducers, producing highest xylanase activities of 150 $U.g^{-1}$ substrate and 15 $U.g^{-1}$ substrate, respectively (Silva et al. 2005). Garcia et al. (2015) cultivated *Lichtheimia ramose* and reported highest activities of β-glucosidase of 162 $U.g^{-1}$ in wheat bran as carbon source and the lowest activity of 0.68 $U.g^{-1}$ was obtained on rice husk. Bajaj et al. (2011) grew *Penicillium* sp. in several lignocellulosic biomasses, testing different carbon sources reporting maximum xylanase activities of 412 $U.g^{-1}$ dry substrate of wheat bran, 436 $U.g^{-1}$ dry substrate of rice bran, and 214 $U.g^{-1}$ dry substrate of sawdust.

In the experimental design using *A. brasiliensis* BLf1 growing on rice husk as substrate in SSC, the lowest value of xylanase activity was obtained when the lowest concentration of basal medium was used (experiment 8). Maximum xylanase activity obtained in the test with highest concentration of basal medium, these results and the values shown in table 7 suggest that the basal medium is essential to provide minerals lacking in the natural substrate to sustain fungal growth and enzymatic activities. Other variables showing influence over microbial growth and enzyme activity were pH and particle size.

Table 7. Statistical analysis of fractional factorial design 2^{5-1}

A. brasiliensis BLf1			
Factor	Effect	Error	p-value
Mean	111.12*	5.40	< 0.05
Linear			
X_1	- 26.03*	10.80	< 0.05
X_2	2.19	10.80	0.84
X_3	-57.01*	10.80	< 0.05
X_4	2.22	10.80	0.84
X_5	50.50*	10.80	< 0.05
Quadratic			
X_1X_2	-4.72	2.24	0.12
X_1X_5	12.56*	2.24	< 0.05
X_2X_3	-23.93*	2.24	< 0.05
X_2X_4	-3.69	2.24	0.20
X_2X_5	-4.62	2.24	0.13
X_3X_4	-11.3*	2.24	< 0.05
X_4X_5	-15.6*	2.24	< 0.05

X1: particle size ; X2: moisture; X3: pH; X4: inoculum size; X5: basal medium

It is interesting, from the point of view of industrial applications, specially concerning food formulations, the possibility of using these fungi strains to produce xylooligosaccharides. Therefore, the culture extracts that were sampled to determine xylanase activities along the experimental design were also analyzed for the presence of XOS.

The production of xylooligosaccharides did take place on the same growth of the production of the enzyme, although at different times, during the cultivation. The highest concentration of XOS was observed at 48 h of cultivation, possibly because

xylan was still present in the culture medium (3.58 $mg.g^{-1}$ substrate of X5 , 4.49 $mg.g^{-1}$ substrate of X4, 6.41 $mg.g^{-1}$ substrate of X2, 48.4 $mg.g^{-1}$ substrate of xylose, at 216 h, 51.3 $mg.g^{-1}$ substrate of xylose). At the end of the cultivation (216 h) there was only xylose in the medium. Figure 4 shows the chromatogram.

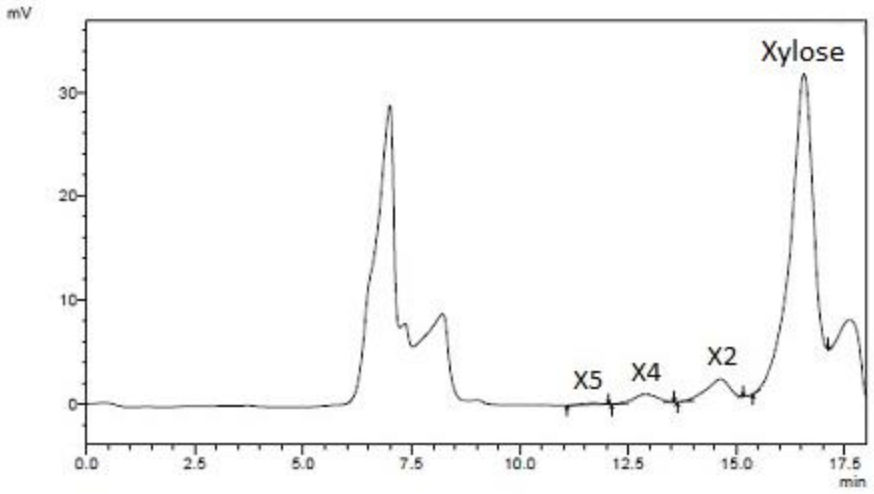

Fig 4. Chromatogram of XOS production in culture extracts obtained in the experimental design (treatment 11, highest xylanase activity), at 48 h of cultivation of *A. brasiliensis* BLf1 growing on rice rusk in SSC; (X2) xylobiose; (X4) xylotretaose; (X5) xylopentose; xylose.

Most of reports on the literature concerning the production of XOS were obtained in submerged cultivations. Although a comparison with these reports is somehow difficult due to the dilution factor and because most of results are presented as volumetric concentrations, our results suggest significant concentrations of XOS, which may still be increased by optimizing the conditions for production in solid-state cultivation (Jayapal et al. 2013; Brienzo et al. 2010; Kocabas and Ozben 2014). Additionally, SSC presents the advantage to allow high-concentration recovery of XOS

from the fermented medium by percolation using appropriate buffers and solutions, which is highly desirable from the industrial point of view.

Conclusions

The screening of new strains of fungi to produce lytic enzymes to be used in the preparation of lignocellulosic hydrolysates was carried out testing three abundant agro-residues as substrates, both in solid-state and submerged cultivations. All substrates were effective in inducing enzymatic activities, with one strain of *Aspergillus brasiliensis* BLf1 showing the highest activities for all enzymes, except for cellulases in SSC. The xylanase activities were optimized for some important variables when considering SSC, showing to be influenced by the basal medium, pH, and particle size of the substrate. Although not optimized in this work, it was possible to demonstrate the production of xylooligosaccharides by *A. brasiliensis* BLf1 on rice husks, showing a good profile of XOS that could be used as functional foods.

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CAPÍTULO III: COMPARATIVE PRODUCTION OF XYLANASE AND THE LIBERATION OF XYLOOLIGOSACCHARIDES FROM LIGNOCELLULOSIC BIOMASS BY *ASPERGILLUS BRASILIENSIS* BLF1 AND RECOMBINANT *ASPERGILLUS NIDULANS* XYNC A773

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Comparative production of xylanase and the liberation of xylooligosaccharides from lignocellulosic biomass by *Aspergillus brasiliensis* BLf1 and recombinant *Aspergillus nidulans* XynC A773

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Abstract

We investigated the simultaneous production of xylanase and the liberation of xylooligosaccharides in rice husk solid-state cultivations of *Aspergillus brasiliensis* BLf1 and by the recombinant *Aspergillus nidulans* XynC A773 strain. The bioprocess was optimized by experimental fractional design and response surface analysis. Results show that both fungi strains produced xylanases and their activities were dependent on the addition of basal medium, moisture content, and the interactions between particle size and inoculum size, producing maximum xylanase activities of 230.7 $U.g^{-1}$ substrate for *A. brasiliensis*, and 187.9 $U.g^{-1}$ substrate for *A. nidulans* XynC. Xylooligosaccharides were liberated in the same cultures, with concentrations up to 17.6 $mg.g^{-1}$ and 23.9 $mg.g^{-1}$ of substrate for *A. brasiliensis* and *A. nidulans* XynC, respectively, both strains presenting similar profiles, with xylose residues varying from X3 to X6. These results suggest the possibility of lowering production costs of enzymes for applications and oligosaccharides.

Keywords: *Aspergillus* fungi; lignocellulosic biomass; xylanase; xylooligosaccharides; rice husk; prebiotics production.

Introduction

Agro-industrial lignocellulosic residues are now considered fermentation substrates in bioprocesses, helping to reduce industrial costs and being important alternatives to solve environmental problems caused by their accumulation in nature (Alvira *et al.*, 2010). These materials are basically sugar polymers, but their lignocellulosic nature have complex structures, consisting mainly of cellulose, hemicellulose, and lignin, and physico-chemical or enzymatic treatments are required to liberate their fermentable sugars (Sun *et al.*, 2014). For the liberation of xylose and its polymers, in particular, it is required the action of xylanases and xylosidases. This treatment can be accomplished by fungi cultivations, which are able to use xylan-rich residues of hemicellulose of various biomass, such as wheat bran, rice straw, and corn cob (Anthony *et al.*, 2003; Qinnghe *et al.*, 2004). Rice husk, the outer layer of the paddy grain, is readily available in bulk quantities as a by-product of rice processing industries. This agro-industrial waste is known to contain carbohydrate polymers such as cellulose, hemicellulose, and xylan, as well as arabinose and it has been used in some research as an ingredient in the preparation of xylooligosaccharides (XOS), showing properties referred to as promising prebiotics (Aderolu *et al.*, 2007).

Xylanases hydrolyze the internal glycosidic bonds along the xylan chain, releasing XOS of various degrees of polymerization. XOS composed of 2 to 4 sugar units are ideal to be used in foods, because of their prebiotic properties (Gullón *et al.*, 2011b; Loo *et al.*, 1999; Knob *et al.*, 2010; Saha, 2003).

XOS are considered prebiotics because of their potential action upon human physiology, being selectively fermented by some types of intestinal bacteria, mainly probiotic bacteria, such as several species of bifidobacteria and lactobacilli. XOS are

processed in the large intestine, stimulating the production of short chain fatty acids, favoring host health (Gullón *et al.*, 2011b).

Both xylanase and XOS productions have been mainly accomplished by the use of filamentous fungi, including recombinant strains (Kocabas & Ozben, 2014). In this respect, *Aspergillus nidulans* represents a tractable laboratory model system, to produce and secrete large amounts of heterologous enzymes and in previous work of our group, the genes encoding the *Penicillium funiculosum* endo-xylanase (XynC) were transferred to *A. nidulans* for improved xylanase activities (Gonçalves *et al.*, 2012).

In this context, in the present work we evaluated the xylanase production by the fungus *Aspergillus brasiliensis* BLf1, isolated by us from the Amazonian environment and never used in bioprocess before, and by the recombinant fungus *Aspergillus nidulans* XynC A773, using rice husk – a rich source of xylan - as substrate in solid state cultivations (SSC). Important culture variables were optimized using experimental fractional design and response surface analysis. In the same cultivation system, we investigated the liberation of XOS and analyzed their degree of polymerization to infer on their prebiotic properties.

Materials and methods

Microorganisms and their maintenance

The strain *Aspergillus brasiliensis* BLf1 was isolated from the Amazon environment and it is deposited in the culture collections of the BiotecLab Laboratory (ICTA, UFRGS, Porto Alegre, Brazil). The complete description of strain isolation and genetic analysis and characterization can be found in Menezes *et al.* (2017). This strain was chosen because in a previous work it has shown to efficiently hydrolyze lignocellulosic biomass (rice husk, soybean hull, and spent malt), and to produce several

enzymes of biotechnological interest, among them xylanase (Menezes *et al.*, 2017). The strain *Aspergillus nidulans* XynC A773 was provided by the National Laboratory of Bioethanol Science and Technology (CTBE, CNPM, Campinas, SP, Brazil), being a recombinant strain, which was genetically modified by transformation with vector pEXPYR for the expression and secretion of hemicellulolytic enzymes (Segato *et al.*, 2012). The gene encoding endo-xylanase (XynC) of *Penicillium funiculosum* was transferred to *Aspergillus nidulans* strain A773. The coding sequence for the XynC gene, along with further information on plasmid construction and cell transformation can be found in Segato *et al.* (2012).

A. brasiliensis BLf1 was maintained on potato dextrose agar (PDA) and *A. nidulans* XynC A773 was maintained on minimal medium (MM) supplemented with pyridoxine (Segato *et al.*, 2012), and kept at 4 ºC.

Agro-industrial residue used in the solid-state cultivations

Rice husk was chosen as the substrate for the solid-state cultivation based on its chemical composition and xylan content (Menezes *et al.*, 2017). This biomass was provided in its natural form, without any previous thermal or physical treatment, by Extremo-Sul Company (Camaquã, RS, Brazil, 30º 51' 04" S; 51º 48' 44" W). In the laboratory, the material was milled using a knife mill to a particle size of 1 mm and 2 mm in diameter and the processed material was stored in sealed plastic bags and kept at room temperature until experimentation.

Solid-state cultivations

The solid-state cultivations of fungi were carried out using 250 mL Erlenmeyer flasks containing 5 g of substrate. Basal medium was added to the substrate in order to

achieve desired moisture content (60 and 80 %), and it was formulated according Segato *et al.* (2012): 6 $g.L^{-1}$ $NaNO_3$; 0.52 $g.L^{-1}$ KCl; 0.52 $g.L^{-1}$ $MgSO_4.7H_2O$; 1.52 $g.L^{-1}$ KH_2PO_4; 1 $g.L^{-1}$ pyridoxine, 2 mL of salt trace solution (containing in $g.L^{-1}$): 22 $ZnSO_4.7H_2O$; 11 H_3BO_3; 5 $MnCl_2.4H_2O$; 5 $FeSO_4.7H_2O$; 1.6 $CoCl_2.6H_2O$; 1.6 $CuSO_4.5H_2O$; 1.1 $(Mn_4)_6Mo_7O_{24}$; and 5 EDTA. Sodium phosphate and citrate buffer was used in order to achieve pH 7.5 and 4.5, respectively. The flasks were sterilized by autoclaving at 121 °C for 20 min, and inoculated with either 1.10^5 or 1.10^8 spores per gram of substrate (according to the experimental design, see below), measured by cell counting using the Neubauer chamber counting. The culture flasks were incubated for 9 days at 37 °C.

Experimental design

A fractional factorial experimental design 2^{5-1} was performed to obtain the best conditions for the activity of the xylanase enzyme. The variables and their coded and uncoded values are presented in Table 1, whereas in Table 2 are shown the 16 treatments obtained for the five variables, each at two levels. The design was constructed of sixteen factorial points and in each case, the xylanase activity was determined after five days of cultivation.

Preparation of extracts for xylanase activity analysis and determination of XOS

After cultivation, the crude enzymatic extract of cultures were prepared following procedures described by Menezes *et al.* (2017). Reducing sugars liberated by hydrolysis of these substrates were quantified using the dinitrossalicylic method (Miller 1959). The same extract was used without further treatments to evaluate xylanase activities and the amount and profile of XOS liberated in the process. The

xylooligosaccharides were determined by HPLC, using an Aminex HPX 42 column, Bio Rad, under the following conditions: column temperature was 85 ^{o}C, using ultra-pure water as eluent with the flow 0.6 ml.min^{-1} and a refractive index detector (Menezes *et al.*, 2017).

Statistical analysis

The experimental design and analysis of results were carried out using Statistica 12.0 (Statsoft, USA). Statistical analysis of results and the model was performed as analysis of variance (ANOVA) at $p < 0.05$.

Results and discussion

Experimental design of SSC of *A. brasiliensis* BLf1 and *A. nidulans* XynC A773

Because we were testing a very recalcitrant agro residue, rice husks, it was important to determine some variables that would impact cell growth and metabolism and, consequently, the enzymatic activity, of the solid-state cultivation of fungi. Five variables were studied by experimental design for the cultivation of *A. brasiliensis* BLf1 and *A. nidulans* XynC A773 (Table 1), and the coded variables and the fractional factorial design 2^{5-1} results for both fungi are shown in Table 2. Table 3 presents statistical significance of each variable in the production of xylanase, whereas Figure 1 shows the interaction of particle size and inoculum size, and inoculum size and basal medium.

Table 1 Variables and their different levels used in the fractional factorial experiment for both fungi.

Variables	Name	Coded levels	
		-1	+1
X_1	Particle size (mm)	1	2
X_2	Moisture (%)	60	80
X_3	pH	4.5	7.5
X_4	Inoculum size (spores.g^{-1})	10^5	10^8
X_5	Basal medium (mL)*	0	4

(*) Basal medium composition in M&M.

Table 2 Experimental design and the results of fractional factorial design 2^{5-1}

Treatment	Levels					Xylanase Activity ($U.g^{-1}$ dry substrate)	
	X_1	X_2	X_3	X_4	X_5	*A. brasiliensis* BLf1	*A. nidulans* XynC A773
1	-1	-1	-1	-1	1	139.1	71.9
2	1	-1	-1	-1	-1	40.4	8.5
3	-1	1	-1	-1	-1	28.9	40.1
4	1	1	-1	-1	1	215.3	92.9
5	-1	-1	1	-1	-1	31.1	3.0
6	1	-1	1	-1	1	217.1	80.8
7	-1	1	1	-1	1	228.2	187.9
8	1	1	1	-1	-1	21.5	2.8
9	-1	-1	-1	1	-1	79.4	6.7
10	1	-1	-1	1	1	189.3	94.4
11	-1	1	-1	1	1	230.7	157.8
12	1	1	-1	1	1	51.2	26.3
13	-1	-1	1	1	1	217.7	68.2
14	1	-1	1	1	-1	40.4	6.7
15	-1	1	1	1	-1	40.0	23.8
16	1	1	1	1	1	178.9	186.3

* X1: particle size ; X2: moisture; X3: pH; X4: inoculum size; X5: basal medium

Table 3 Statistical analysis of fractional factorial design 2^{5-1}

B. brasiliensis BLf1				*A. nidulans* XynC A773			
Factor	Effect	Error	p-value	Factor	Effect	Error	p-value
Mean	125.2*	1.8	< 0.05		66.1*	5.8	< 0.05
Linear							
X_1	- 11.8	9.8	0.05	X_1	-7.6	15.6	0.22
X_2	-1.7	9.8	0.68	X_2	47.2*	15.6	< 0.05
X_3	-6.7	9.8	0.17	X_3	7.6	15.6	0.22
X_4	6.5	9.8	0.18	X_4	10.3	15.6	0.14
X_5	167.2*	9.8	< 0,05	X_5	102.8*	15.6	< 0.05
Quadratic							
X_1X_4	-15.1*	3.7	< 0.05	X_1X_2	-17.7	4.3	0.05
X_1X_5	-5.3	3.7	0.25	X_1X_4	21.8*	4.3	< 0.05
X_2X_3	-7.7	3.7	0.13	X_2X_3	13.3	4.3	0.09
X_2X_5	10.7	3.7	0.06	X_2X_4	7.3	4.3	0.23
X_3X_4	-11.7	3.7	0.05	X_2X_5	30.17*	4.3	< 0.05
X_3X_5	10.0	3.7	0.07	X_3X_4	-7.6	4.3	0.21
X_4X_5	-15.7*	3.7	< 0.05	X_3X_5	18.9*	4.3	< 0.05
				X_4X_5	8.0	4.3	0.20

* Statistically significant at 95% confidence level. X1: particle size; X2: moisture; X3: pH; X4: inoculum size; X5: basal medium

The test applied for each of the factors in cultures of *A. brasiliensis* BLf1 showed that the linear effect of concentration of the basal medium (X_5) is statistically significant at the 95 % confidence level (ANOVA), being an important variable for xylanase production. The other important variables affecting xylanase activity positively were granulometry and inoculum size. Under best combination of these variables, maximum xylanase activity achieved was 230.7 $U.g^{-1}$ dry substrate.

For *A. nidulans* XynC A773, xylanase activities were highest (187.9 $U.g^{-1}$ dry substrate) when the experiment had a maximum level of basal medium concentration

and moisture, showing that these variables strongly affect xylanase production. Results for both variables were statistically significant at the 95 % confidence level (Table 3). In addition, interactions between particle size and inoculum size, moisture and concentration of basal medium, and finally of pH and concentration of basal medium showed significant quadratic effects on the xylanase response. Therefore, the response surface methodology was applied to determine the best conditions of variables interactions (Figure 2). The highest concentrations of basal medium and of moisture level had a positive effect on enzyme activity (Figure 2B), whereas basal medium is independent of pH (Figure 2A). The interaction of particle size and inoculum size are of a more complex nature (Figure 2C).

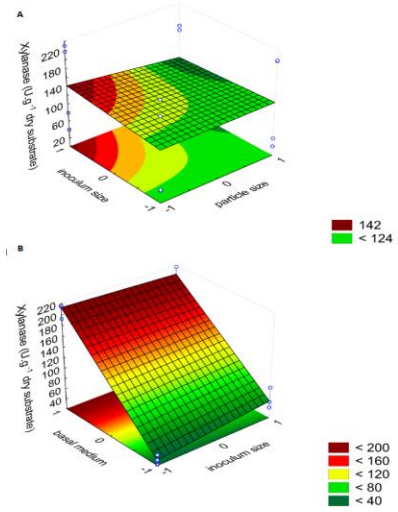


Fig. 1 Response surface of experimental design of *Aspergillus brasiliensis* BLf1 of the interaction between (A) particle size and inoculum size; and (B) inoculum size and basal medium.

Smaller particle size required a smaller inoculum size, possibly because the compaction of material would ease microbial population of the surface, and because of better moisture stability; likewise, larger substrate particle was more efficiently colonized when a larger inoculum was used. However, a visual and microscopic

analysis of fungi morphology of the cultures did not show any differences between the fungi.

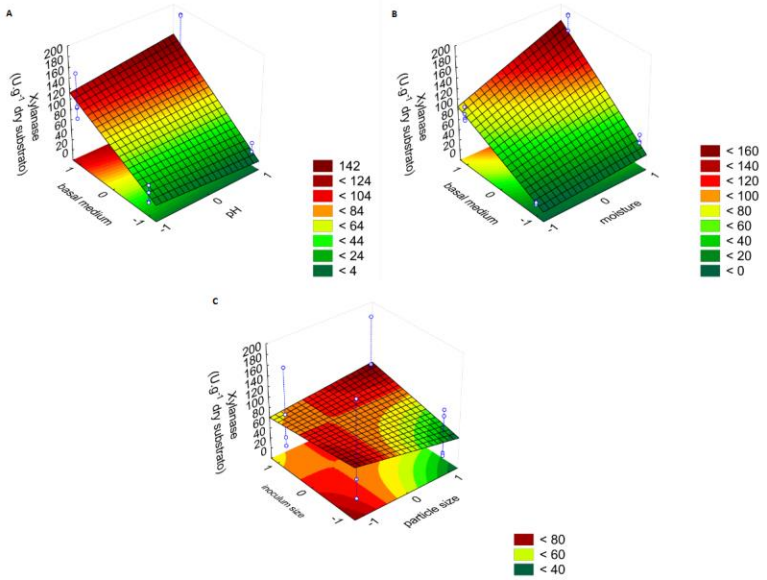


Fig. 2 Response surface of experimental design of *Aspergillus nidulans* XynC A773 of the interaction between (A) pH and basal medium; (B) moisture and basal medium; and (C) particle size and inoculum size.

Although *A. nidulans* XynC A773 was genetically modified for the overexpression of xylanase, it showed lower enzymatic activity than *A. brasiliensis* BLf1 under same experimental conditions, suggesting that the adaptation of the later to more harsh cultivation conditions, such as in SSC, is efficient for the enzyme production. *A. nidulans* XynC A773 was cultivated in submerged cultures by Segato *et al.* (2012), who reported activities of 301.2 $U.mg^{-1}$ protein, assayed with arabinoxylan as substrate. These results are not comparable because of substrate and culture conditions, but they show ability of this strain to secrete large amounts of enzyme.

SSC validation for xylanase production by A. brasiliensis BLf1 and A. nidulans XynC A773

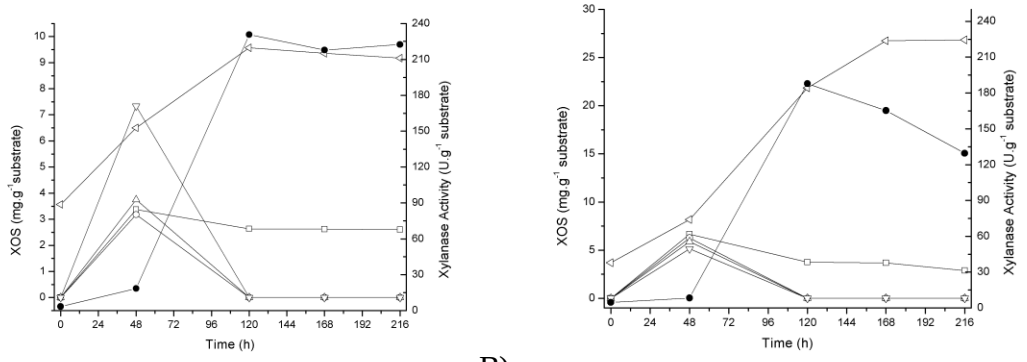
Experimental validations of SSC were conducted under the optimized variables values to confirm the best conditions for enzyme production by both fungi, which were run with five experimental replications. The average xylanase activities obtained were 233.1 ± 2.0 $U.g^{-1}$ dry substrate for *A. brasiliensis* BLf1, and 188.3 ± 0.98 $U.g^{-1}$dry substrate for *A. nidulans* XynC A773, respectively, showing excellent correlations with the statistical design (Table 2).

Although results of enzymatic activities are not readily comparable among different works, the results obtained in our research compared well with the production of xylanase reported by similar bioprocesses. Ghoshal *et al.* (2015) studying the production of extracellular 1,4-β endo xylanase in solid state fermentation on corn cobs, wheat bran, rice bran, rice straw, and wheat straw, and sugarcane bagasse by *Penicillium citrinum* MTCC 9620, reported highest xylanase activities of 52.8, 123.5, 93.2, 31.5, 60.1, and 156.5 $U.g^{-1}$ dry substrate, respectively. The variability of results show that the enzymatic activities are related to substrate composition, physical structures, and the capacity to absorb water in SSC. The production of xylanase by several *Aspergillus* species has been reported in some studies. Maciel *et al.* (2008) produced xylanase by *Aspergillus niger* LPB 326 cultivated on lignocellulosic substrate composed of sugarcane bagasse and soybean meal (which is not a lignocellulosic residue but rather a proteic substrate) under SSC, reporting enzymatic activities as high as 3,099 $U.g^{-1}$ of dry substrate. Pérez-Rodrigues *et al.* (2014) run SSC of *A. niger* CECT and corncob as substrate, obtaining 138 to 500 $U.g^{-1}$ dry substrate. Contrasting

with these results, Santos *et al.* (2013) reported activities as low as 59.3 $U.g^{-1}$ of cocoa (whole fruit), using *A. niger*.

Production of xylooligosaccharides

The same SSC for enzymatic production were evaluated for the liberation of XOS, in order to determine whether the same bioprocess could be used to obtain enzyme and the products of the enzymatic activity, in an attempt to turn the process more economically attractive. Figure 3 shows the results for the evolution of XOS along nine days of SSC, (experiments 11 and 7 for *A. brasiliensis* BLf1 and *A. nidulans* XynC A773, respectively, Table 2). The highest XOS concentration, considering the sum of X3 to X6, was observed at 48 h of cultivation, whereas the highest xylanase activity was detected at 120 h. The chromatograms showing the profile of XOS production at their highest concentrations are shown in Figure 4.



A) B)

Fig. 3 Evolution of XOS along nine days of SSC, same experiment for enzyme production (A) Experiment 11 with *A. brasiliensis* BLf1, and (B) Experiment 7 with *A. nidulans* XynC A773; Symbols: □ xylotriose; ○ xylotretaose; △ xylopentose; ▽ xylohexose; ◁ xylose; ● xylanase activity.

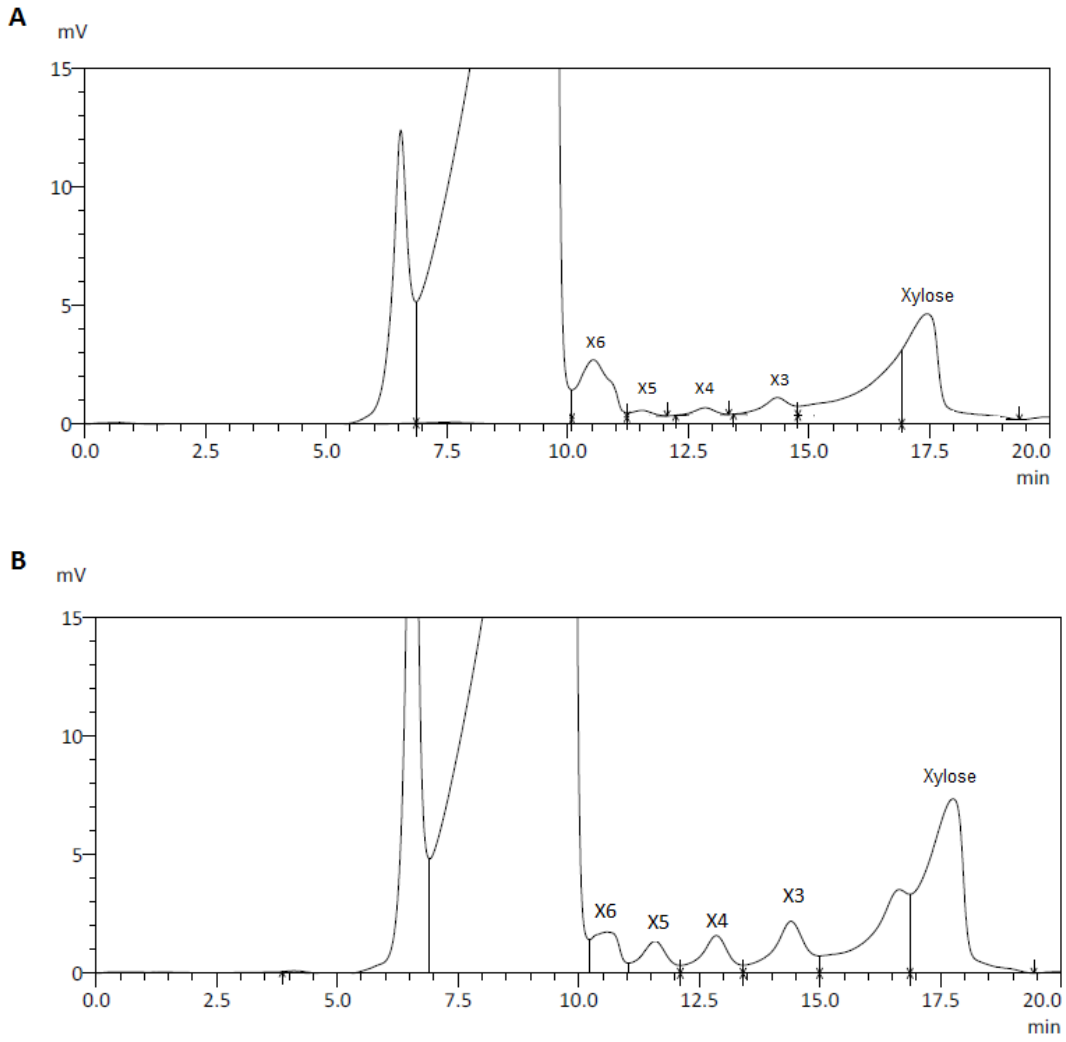


Fig. 4 Chromatogram of XOS production obtained in the experimental design at 48 h of cultivation with; (A) *Aspergillus brasiliensis* BLf1 (treatment 11, Table 1), and (B) *Aspergillus nidulans* XynC A773 (treatment 7, Table 1); (X3) xylotriose; (X4) xylotretaose; (X5) xylopentose; (X6) xylohexose.

Both fungi produced the same degree of polymerization of XOS, but their concentrations varied. In the cultivation using *A. brasiliensis* BLf1, only xylohexose (X6) appears in high concentration, 7.3 $mg.g^{-1}$ substrate. Although the xylanase production by *A. nidulans* XynC A773 was lower than that observed for *A. brasiliensis* BLf1 strain, its general XOS production was higher in the SSC: *A. nidulans* XynC A773

strain produced 23.9 mg XOS.g^{-1} substrate, whereas *A. brasiliensis* BLf1 produced 17.6 mg XOS.g^{-1} substrate (Table 4). It is important to note that the optimization conditions sought in this work were those for enzyme activity, which could differ from best conditions for XOS liberation. Moreover, most of research on XOS production relies on a two-step process, where the enzyme is produced, either by SSC or submerged cultivation, purified and then applied to an appropriate substrate, whereas in this work we carried out both steps in a single process.

Table 4 Maximum concentrations of XOS (X3 to X6), and xylose, obtained in the SSC of *A. brasiliensis* BLf1 and *A. nidulans* XynC (48 h of cultivation).

	Concentrations (mg.g^{-1} substrate)	
	A. brasiliensis BLf1	*A. nidulans* XynC A773
X3	3.4	6.6
X4	3.2	6.3
X5	3.7	5.9
X6	7.3	5.1
Xylose	6.5	8.2
Total XOS*	17.6	23.9

(*) Does not include xylose.

As expected, the kinetic profile shown in Figure 3, shows the conversion of XOS from higher to lower degree of polymerization as time advances, xylose being the only product at the end of 120 h.

Because the XOS released in the solid cultivation are concentrated when extracted using buffer, they can be more easily purified compared to XOS produced in

submerged cultures, which are diluted in the medium. This is another important vantage of the process in our work.

Although it is not possible to directly compare our results with those reported by other authors in terms of concentration of XOS, we present some qualitative comparisons. Gonçalves *et al.* (2012), tested *A. nidulans* XynC A773 for XOS production without quantification, but the authors evaluated the peaks of liberated XOS by capillary electrophoresis of samples of submerged cultures containing sugarcane bagasse as substrate, and observed a profile similar to the one obtained in this study, with presence of X2 to X6. It is important to highlight that the authors pretreated sugarcane bagasse to expose xylose-containing polysaccharides, therefore granting the enzymatic accessibility to the substrate. Concerning the degree of polymerization, Jayapal *et al.* (2013), used xylan extracted from sugarcane bagasse and applied a commercial endoxylanase from *Trichoderma viridae* (Sigma, USA), obtaining 1.15 $mg.mL^{-1}$ of X2, 0.57 $mg.mL^{-1}$ of X3, and 1.15 $mg.mL^{-1}$ xylose. Although not directly comparable with our results based on different substrates and enzymes, the profile of XOS produced in this work using rice husk under SSC varied in degree of polymerization from X2 to X6, which is considered to have a better functionality as food ingredient (Giese *et al.*, 2011). Brienzo *et al.* (2010), obtained the maximum XOS concentration of 5.8 $mg.mL^{-1}$ at 96 h, working with xylan extracted from sugarcane bagasse and using xylanase (60 $U.g^{-1}$ hemicellulose), obtained by submerged culture of *Thermoascus aurantiacus*, obtaining an XOS profile consisting of xylose, X2, X3 and X5, with 60% corresponding to X2 and xylose.

XOS may be present in human food or may be added to industrial products (Nabarlatz *et al.*, 2007). XOS with degree of polymerization (DP) between 2 and 5 are preferred in the production of functional foods because they are consumed by probiotic

bacteria (Kiran *et al.*, 2013). However, when they present a profile with xylobiose (X2), xylotriose (X3) and xylotetraose (X4), they present higher prebiotic effect (Gullón *et al.*, 2011a). Therefore, we can conclude that these oligomers produced from rice husk by the two strains of *Aspergillus* fungi used in this work may have functional action and applicability in food products, based on their degree of polymerization varying from 3 to 6.

Current population lifestyles, including inadequate food intake and quality (Farias and Lopes, 2004), makes difficult to maintain a healthy intestinal microbial community. An adequate diet, with the consumption of prebiotic fibers, can bring benefits to the health of the host, through the balance of the intestinal microbiota, mainly composed of probiotic bacteria such as B*ifidobacterium* and *Lactobacillus* (Clemente *et al.*, 2012). Xylooligosaccharides used as functional food depending on the degree of polymerization, may be fermented by these bacteria and selectively stimulate the growth or activity of one or a limited number of these probiotic bacteria, which is known as the prebiotic effect (Gibson, 2008; Vandenplas, 2011).

We performed a study to facilitate the production of XOS in the same culture of enzyme production, with the effect to lower cultivation time and of production costs, using the rice husk, which has not been pretreated, contrasting with most of other studies, in which residues are physico-chemically pretreated (Jayapal *et al.*, 2013; Xue *et al.*, 2016; Azelee *et al.*, 2016).

Conclusions

Results showed the possibility of simultaneously producing xylanases and food prebiotics (XOS) using rice husks, an inexpensive lignocellulosic biomass, under the conditions of solid-state cultivation of GRAS fungi. Production of xylanases by *A. brasiliensis* BLf1 and *A. nidulans* XynC A773 are efficient to be used in bioprocesses

using rice husk, and possibly other biomass, as substrate. The enzyme activities were influenced by the variables basal medium and moisture, as well as interactions between granulometry and inoculum size. Additionally, the production of XOS from rice husk was technically feasible, with reasonable concentrations of XOS being released in the medium, showing varying degrees of polymerization, which is highly interesting for food applications. Biological reaction time to obtain XOS was comparatively short (48 h), compared to other reports. Further studies are granted to investigate the use of different lignocellulosic residues in this process, and to optimize the reaction conditions for XOS production.

Conflict of interest

The authors declare no conflict of interest

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CAPÍTULO IV: XYLOOLIGOSACCHARIDES PRODUCTION BY FUNGI CULTIVATIONS IN RICE HUSK AND THEIR APPLICATION AS SUBSTRATE FOR LACTIC ACID BACTERIA GROWTH

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Xylooligosaccharides production by fungi cultivations in rice husk and their application as substrate for lactic acid bacteria growth

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Abstract

We describe a three-steps bioprocess to convert rice husk as source of xylooligosaccharides and then used these prebiotics to grow lactic acid bacteria as a model to produce probiotics from vegetal sources. First, *Aspergillus brasiliensis* BLf and the recombinant *Aspergillus nidulans* XynC A773 strains were solid-state cultivated in rice husk to produce xylanases (234.7 ± 0.01 $U.g^{-1}$ and 192 ± 0.03 $U.g^{-1}$ substrate, respectively). These enzymatic preparations were applied to rice husk to hydrolyse its hemicellulosic structure and obtain xylooligosaccharides (37.25 mg $XOS.g^{-1}$ substrate and 75.92 mg $XOS.g^{-1}$ substrate, respectively). Prebiotic effects of XOS were tested by cultivating probiotic strains *Lactobacillus plantarum* BL011 and *Bifidobacterium lactis* B-12, which grew in this substrate. The results in this work showed that it is possible to produce xylooligosaccharides and then proceed to use these prebiotic sugars to produce probiotic bacteria using the same agro-residue, being possible its use in food related applications.

Keywords: Rice husk; *Aspergillus* sp.; xylanase; xylooligosaccharides; probiotics.

1. Introduction

Xylooligosaccharides (XOS) are xylose oligomers that can be produced by enzymatic hydrolysis of xylan. XOS contain from 2 to10 units of xylose, which are linked by β-(1,4) bonds, and are considered fibers having important nutraceutical properties (Milessi et al., 2015). Although XOS can be obtained via chemical synthesis or by enzymatic synthesis using pure substrates, one alternative for XOS production

would be the enzymatic hydrolysis of biomass materials, making the process more cost-effective and sustainable (Moniz et al., 2014).

Rice (*Oryza sativa*) is one of the most produced and consumed cereals in the world, characterized as the main food for more than half of the world population. Rice husk is the abundant residue of rice processing, of which about 11 % is composed by hemicellulose, in dry basis (Menezes et al., 2017; Saha et al., 2005; Hickert et al., 2013), with xylan being its main sugar polymer (Perez et al, 2005; Walter et al., 2008).

XOS are added-value functional food additives that can be produced from lignocellulosic biomass and the use of rice husk is an economical alternative for its production because this material has a very low or no cost at all, in many cases simply being discarded in the environment (Nara et al, 2014; Samanta et al., 2015). Xylanases (1.4-β-d-xylanases, EC 3.2.1.8) are the main endo-enzymes that hydrolyze randomly β-1,4 linkages of xylans, the main polysaccharide of hemicelluloses (Akin, 2008). Several microbial sources of xylanase, both bacterial and fungal, are used as enzymatic extracts to produce XOS. Some studies also reported on the use of recombinant microorganisms to overexpress genes for xylanase production. The use of a xylanase from *Bacillus subtilis* (XynA), overexpressed in *Escherichia coli*, was tested for XOS production and good results were obtained (Bragatto et al., 2013). Driss et al. (2014) studied the production of xylooligosaccharides obtained from corncobs using *Penicillium occitanis* xylanase. Zheng et al. (2014) characterized a thermostable xylanase from *Paenibacillus* sp. NF1 that could be applied in xylooligosaccharides preparations.

Scientific and commercial interest in oligosaccharides has increased in the last decades due to the identification of several health benefits these compounds can bring to hosts, both in human nutrition and animal feed preparations (Rastall, 2010). In this context, oligosaccharides, in general, show excellent effects and health benefits as

prebiotic when consumed as part of the diet, improving the growth of probiotic microorganisms, such as *Bifidobacterium* and *Lactobacillus*, strains that are found in the human digestive system (Falck et al., 2013). Probiotic microorganisms live in the digestive system of humans and other animals, being responsible for several important mechanisms of nutrient absorption and overall health of the body (Adolfsson et al., 2004). It has been shown that XOS selectively stimulate the growth of these beneficial bacteria in the colon, thus possessing prebiotic properties and could be used in foods as a functional additive (Mäkeläinen et al., 2010; Falck et al., 2013; Reddy and Krishnan, 2016; Jagtap et al., 2017). The efficient and complete metabolization of XOS by these bacteria, requires several enzymes, including β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, and acetyl xylan esterase, thus the ability of probiotic microorganisms to metabolize XOS must be tested (Zeng et al., 2007). In some countries, such as Japan, XOS have been approved for Food for Specified Health Uses (FOSHU), as ingredients for food to improve gastrointestinal conditions (Mäkeläinen et al., 2009).

Considering the potential market demand for XOS to be applied in the food industry, and the growing interest in the health benefits of probiotic bacteria, the present work aimed at studying the possible production of xylooligosaccharides using rice husk as substrate and their subsequent evaluation as prebiotics. We postulated a three-steps approach: first, we produced xylanases by culturing *Aspergillus brasiliensis* BLf1 and the recombinant strain *Aspergillus nidulans* XynC A773 separately, as solid-state cultivation (SSC) in rice husk; then, enzymatic supernatants of fungi SSC were applied to the same substrate in order to liberate XOS. Finally, the prebiotic effect of XOS was evaluated by cultivating the probiotic strains of *Lactobacillus plantarum* BL011 and *Bifidobacterium lactics* B-12 in media containing these sugars.

2. Materials and Methods

2.1 Agro-industrial residue and other materials

The rice husk used in this work was provided by Extremo-Sul Company (Camaquã, RS, Brazil, geo-coordinates 30º 51'' 04' S; 51º 48'' 44' W). The material was milled in a knife mill until mean particles size of 1 mm in diameter was achieved. The processed material was stored in sealed plastic bags and kept at room temperature until experimentation, without any further modifications. The chemical composition of rice husk was published in a previous work (Menezes et al., 2017). All chemicals used in this research were of analytical grade and were purchased from Sigma-Aldrich (São Paulo, Brazil; St Louis, USA).

2.2 Microorganisms

The strain *Aspergillus brasiliensis* BLf1 was isolated from soil samples of the environment by our group and the complete isolation, identification, and characterization of this strain are described elsewhere (Menezes et al., 2017). The recombinant strain *Aspergillus nidulans* XynC A773 was provided by the National Laboratory of Bioethanol Science and Technology (CTBE, CNPM, Campinas, SP, Brazil). This strain was genetically modified by procedures described by Segato et al. (2012). Working stocks of *A. brasiliensis* BLf1 were maintained in potato dextrose agar (PDA), whereas stocks of *A. nidulans* XynC A773 were kept in minimal medium (MM) supplemented with pyridoxine (Segato et al., 2012), and kept at 4 ºC.

The *Lactobacillus plantarum* BL011 strain was isolated by our group and its complete description can be found in Coghetto et al. (2016). Working stocks of *L.*

plantarum BL011 were maintained in 20 % (volume fraction) glycerol suspension, frozen at −20 °C. The commercial *Bifidobacterium lactis* BB-12 strain was kindly provided by Christian Hansen Company (Valinhos, Brazil), kept under the same conditions as for the *Lactobacillus* strain.

All microbial strains used in this work were kept as frozen (-80 °C) and lyophilized stocks in the microbial culture collection of the BiotecLab (ICTA, UFRGS, Porto Alegre, Brazil).

2.3 Xylanase production and extraction

Xylanase production was carried out in solid-state cultivation of the fungi *Aspergillus brasiliensis* BLf1 and *Aspergillus nidulans* XynC A773, cultivated separately, having rice husk as substrate. Sodium citrate buffer was used in order to achieve pH 4.5, and sodium phosphate buffer to achieve pH 7.5, respectively, for cultures of *A. brasiliensis* BLf1 and *A. nidulans* XynC A773, respectively. Cultivations were carried out using 250 mL Erlenmeyer flasks containing 5 g of substrate, added of 15 mL of basal medium, in order to achieve the desired final moisture of 80 % (weight fraction). The basal medium was formulated according to Segato et al. (2012), and contained, in $g.L^{-1}$: 6 $NaNO_3$; 0.52 KCl; 0.52 $MgSO_4.7H_2O$; 1.52 KH_2PO_4; 1 pyridoxine, 2 mL of salt trace solution (containing, in $g.L^{-1}$): 22 $ZnSO_4.7H_2O$; 11 H_3BO_3; 5 $MnCl_2.4H_2O$; 5 $FeSO_4.7H_2O$; 1.6 $CoCl_2.6H_2O$; 1.6 $CuSO_4.5H_2O$; 1.1 $(Mn_4)_6Mo_7O_{24}$; and 5 EDTA. The flasks were sterilized by autoclaving at 121 °C for 20 min and were inoculated with either $1 \bullet 10^8$ spores per gram of substrate from *Aspergillus brasiliensis* BLf1 or $1 \bullet 10^5$ from *Aspergillus nidulans* XynC A773, measured by cell counting using a Neubauer chamber. The culture flasks were incubated for 5 days at 37 °C. Enzymatic extraction was carried out by adding 40 mL of 50 mM sodium

acetate buffer pH 5.0 to the culture. The flasks were homogenized in a shaker (180 rpm, 30 min) and then centrifuged (3,000 x g, 4 ºC). The supernatant was collected and used for further analysis and xylanase activities were evaluated following procedures described by Menezes et al. (2017).

2.4 XOS production by xylanase enzymatic extracts

The production of XOS using rice husk as substrate was performed using the crude enzymatic extract containing xylanase as described above. This extract was concentrated using an Amicon membrane (10KDa, Millipore), followed by centrifugation (7,000 xg, 5 min). The enzymatic hydrolysis reaction and liberation of XOS was carried out following fixed conditions of moisture (80 %; 50 mM sodium acetate buffer), pH (5.0), temperature (50 ºC) and enzyme concentration (100 $U.g^{-1}$ xylan). Reaction time for XOS production varied according to the enzymatic extract used: 3 h of reaction using *A. brasiliensis* BLf1 xylanase supernatant, and 24 h when using *A. nidulans* XynC A773 xylanase supernatant. Final concentration of XOS was determined by HPLC, using an Aminex HPX 42A column (Bio Rad, Brazil) and analysis conditions according to procedures described by Menezes et al. (2017).

2.5 Cultivation of probiotic bacteria using XOS as substrate

Bacterial inocula were prepared as follows. Erlenmeyer flasks (250 mL) containing 50 mL of MRS (Man et al., 1960) were inoculated with 1.5 mL of glycerol stock cultures of *Lactobacillus plantarum* BL011 and were incubated at 37 ºC in a rotary shaker at 180 rpm. *Bifidobacterium lactis* BB-12 cultures were inoculated using 1 mL in the same medium under anaerobic conditions, at 37 ºC in a rotary shaker at 50 rpm. Cultures were grow until optical density (OD) of 1.0 in 600 nm. The cells were

harvested by centrifugation (3,500 x*g* for 15 min at 4ºC) and cell pellet was washed twice with sterile distilled water and resuspended directly into the culture broth. This procedure was used as the standard inoculum preparation for all experiments.

XOS metabolization by *L. plantarum* BL011 and *B. lactis* BB-12 strains was studied by replacing glucose for the XOS mixture obtained as described in item 2.4 (10 $g.L^{-1}$) in the MRS culture media. The media were inoculated with 10 % (volume fraction) of bacteria inocula OD 1.0 and were cultured at 37 ºC, initial pH 6.0. Bacteria growth, XOS consumption, and other metabolites were monitored along 96 h (*L. plantarum* BL011) or 120 h (*B. lactis* BB-12). To determine dry cell mass, 5 mL samples of culture broths were centrifuged (5,000 xg for 15min), cell pellet was washed with sterile distilled water in order to measure the microbial biomass by dry weight, using a moisture analyzer equipped with an infrared heating source and an analytical balance (Ohaus, MB25, USA). Controls were made using medium in which there was no carbon source added, or using glucose, xylose, or lactose. All the experiments were carried out in duplicates.

2.6 Other analytical procedures

Because it was expected that lactic acid bacteria would produce organic acids, lactic and butyric acids concentrations in culture samples were also determined by HPLC (Shimadzu, Japan) equipped with a refractive index detector and Bio-Rad HPX 87-H column (300 x 7.8 mm) using 5 mM sulphuric acid as eluent at 45 ºC, a 0.6 $mL.min^{-1}$ flow rate and using sample volumes of 20 µL.

3. Results and Discussion

3.1 Xylanase production in SSC of *A. brasiliensis and A. nidulans*

A. brasiliensis BLf1 SSC in rice husk as substrate resulted in a maximal xylanase supernatant activity of 234.7 ± 0.01 $U.g^{-1}$ dry substrate, whereas *A. nidulans* XynC A773 produced a maximal enzyme supernatant activity of 192 ± 0.03 $U.g^{-1}$ dry substrate. The substrate was effective in inducing enzymatic production of these strains, and the supernatants containing xylanases were used for XOS production. *Aspergillus brasiliensis* BLf1 has proved to be a good xylanase producer using several biomasses as substrates in solid-state cultivation (Menezes et al., 2017), and in the present study, it was possible to obtain highest enzyme yields for this strain so far, using rice husk. The results obtained using strains *A. brasiliensis* and *A. nidulans*, are comparable to other fungi, reported as good producers of xylanase. For instance, Pérez-Rodríguez et al. (2014) reported xylanase activities from 78 to 552 $U.g^{-1}$ dry substrate at 120 h cultivation in the solid-state cultivation of *A. niger* on corncob residue. Gomes et al. (2016), reported xylanases activities of 180 $U.g^{-1}$dry substrate after 168 h of growth of *A. fumigatus* in solid-state cultivations using sugarcane bagasse.

3.2 XOS production using the enzymatic xylanase supernatants from fungi SSC

After extraction of xylanases supernatants produced in the SSC of fungi, these preparations were added to untreated rice husks (see section 2.4) in order to obtain the XOS from this biomass.

The profiles of obtained XOS produced by xylanase of *A. brasiliensis* BLf1 and *A. nidulans* XynC, at their highest concentrations during hydrolysis are presented in Figures 1A and 1 B, respectively, and the comparison between the two fungi in Figure

2. XOS liberation varied markedly for the two enzyme extracts: the extract from *A. brasiliensis* BLf1 was able to release the highest XOS concentrations (18.13 $mg.g^{-1}$ substrate of X3, 9.20 $mg.g^{-1}$ substrate of X4, 7.05 $mg.g^{-1}$ substrate of X5, 2.87 $mg.g^{-1}$ substrate of X6, and 68.5 $mg.g^{-1}$ substrate of xylose, Figure 1A) in a very short reaction time of 3 h, whereas for the *A. nidulans* XynC, the same amount of enzyme took a 24 h reaction to liberate its maximum XOS concentrations (38.57 $mg.g^{-1}$ substrate of X3, 22.19 $mg.g^{-1}$ substrate of X4, 11.40 $mg.g^{-1}$ substrate of X5, 3.76 $mg.g^{-1}$ substrate of X6, and 33.69 $mg.g^{-1}$ substrate of xylose, Figure 1B).

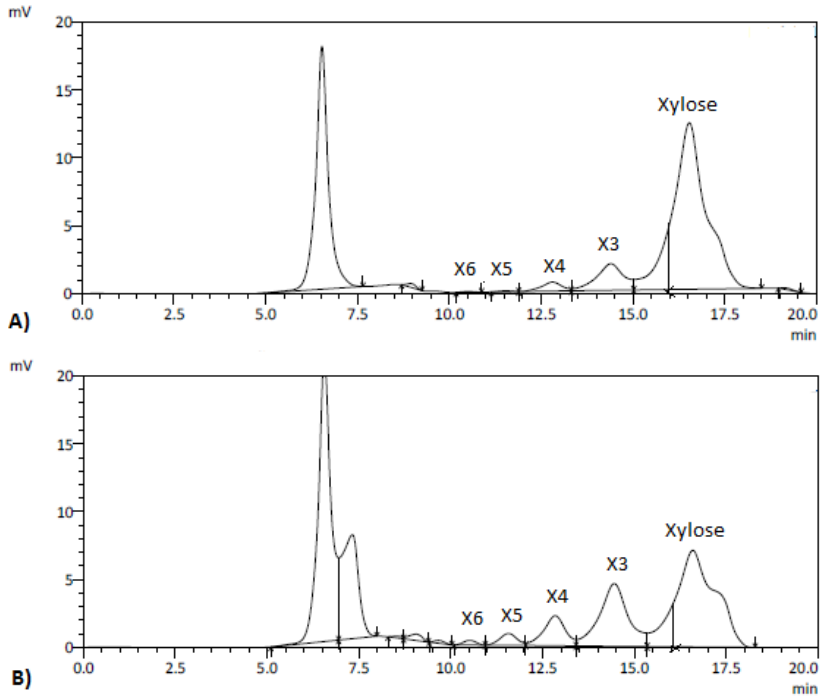


Fig. 1. Chromatogram of the obtained XOS by the application of the enzymatic extracts of (A) *Aspergillus brasiliensis* BLf1 and (B) *Aspergillus nidulans* XynC A773 over rice husk at their maximum concentrations; (X3) xylotriose; (X4) xylotretaose; (X5) xylopentose; (X6) xylohexose.

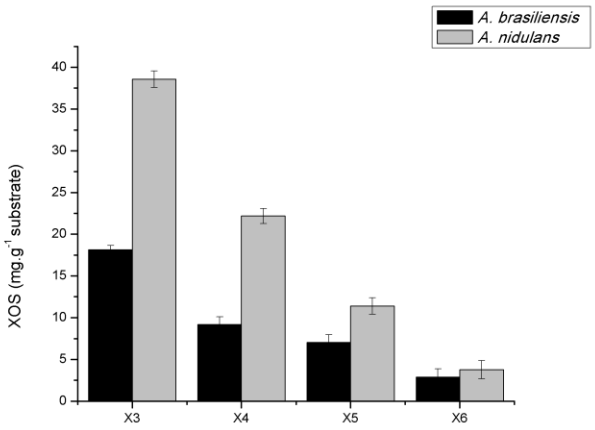
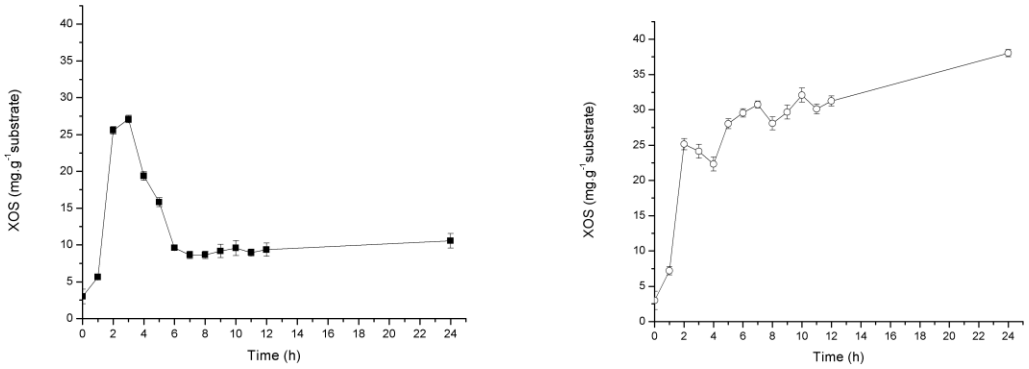


Fig. 2. Comparison of XOS liberated from rice husk by *Aspergillus brasiliensis* BLf1 (black) and *Aspergillus nidulans* XynC A773 (grey) enzymatic extracts. Results are the mean of duplicate kinetics.

In summary, xylanase supernatant of *A. nidulans* XynC A773 strain produced 75.92 mg XOS.g^{-1} substrate at 24h, compared to 37.25 mg XOS.g^{-1} substrate for *A. brasiliensis* BLf1 extract at 3h, under the same conditions . The differences in enzyme titer and time to maximum production can be explained by the fact that *A. nidulans* A773 was genetically modified by transformation using the pUC19 based *E. coli* expression vector pEXPYR. The heterologous gene inserted into the vector were an endo-1,4-xylanase from *P. funiculosum*, bearing the expression gene xynC (Segato et al., 2012; Gonçalves et al., 2012). Therefore, the xylanses from the two fungi must be isoenzymes, with possible differences in their activities. These observations also explain for the difference in time for the maximum XOS liberation in the SSC of these strains (Figure 3).



A B

Fig. 3. Kinetics of XOS liberation from rice rusk by the enzymatic extracts of *Aspergillus brasiliensis* BLf1 (A) and *Aspergillus nidulans* XynC A773 (B) SSC. Results are the mean of duplicate kinetics.

Although it is not possible to directly compared results obtained in this work with others in the literature because of differences in methodologies, some comparison is shown in order to demonstrate the importance of our findings. In the enzymatic hydrolysis of xylan-rich wheat husk, used without any prior pretreatment, using the crude enzymatic extract of *A. fumigatus* R1 (75 $U.g^{-1}$ xylan), after 12 h of reaction unquantified amounts of xylobiose, xylotriose, and xylotetraose were obtained (Jagtap et al., 2017). The enzymatic hydrolysis of oat xylan using the thermostable xylanase from *Paenibacillus* sp. NF1 (30 $U.g^{-1}$ xylan), produced xylooligosacharides of 2 to 4 degrees of polymerization after 2 h of hydrolysis, the majority of them was xylobiose (Zheng et al., 2014). Carvalho et al. (2015) produced xylanase from *A. fumigatus* M51 and used 500 $U.g^{-1}$ xylan over the hemicellulose extracted from sugarcane bagasse, obtaining an amount of XOS of 52 $mg.g^{-1}$ substrate, with polymerization degree 2 to 4, after 3 h of hydrolysis. Steam pre-treated wheat straw was depolymerized to produce 89 mg of XOS per gram of substrate, which had a polymerization degree of 2 to 6, using

7.2 $U.mL^{-1}$ of commercial endo-xylanase (NS50030) and β-glucosidase (Novozym 188), after 24 h of hydrolysis (Álvarez et al. 2017).

3.3 Test of XOS as substrate for lactic acid bacteria growth

It would be important to test whether XOS obtained from rice husk could sustain probiotic bacteria growth. Therefore, we used the two XOS preparations as substrate for *L. plantarum* (Figures 4A to 4D) and *B. lactis* (5A to 5B), and compared the growth against other simple sugars, normally well metabolized by these strains. Both bacteria were able to metabolize XOS and their growth on this substrate was similar with that for other sugars tested (The control without sugar did not show any growth). As expected, the best growth was observed in lactose, the preferential sugar of lactic acid bacteria, with approximately 60 % being consumed in the tested kinetics, compared to 30 to 50 % of XOS metabolization.

The main metabolites associated with *L. plantarum* and *B lactis* are lactic acid and, in the case of *B. lactis*, also butyric acid. The production of lactic acid is a result of the fermentation process by lactic acid bacteria, possessing acidifing, flavouring, and preservative properties in food (Altaf et al., 2005). Lactic acid is also responsible for reducing the population of pathogens in the host gut (Saad, 2006). On the other hand, it is known that *Bifidobacteria* liberates a number of short-chain fatty acids (e.g. acetic acid and butyric acid), which decrease the pH in the gut of hosts. Subsequently, a low pH environment inhibits the growth of pathogens and increases minerals (magnesium and calcium) absorption (Aachary and Prapulla, 2011). In this work, both bacteria showed production of lactic acid (and also butyric acid for *B.* lactis) growing in XOS

and this production was comparable to that obtained in cultures using lactose, confirming the possibility of using these sugars as prebiotics (Figures 4C, 4D, 5C, 5D).

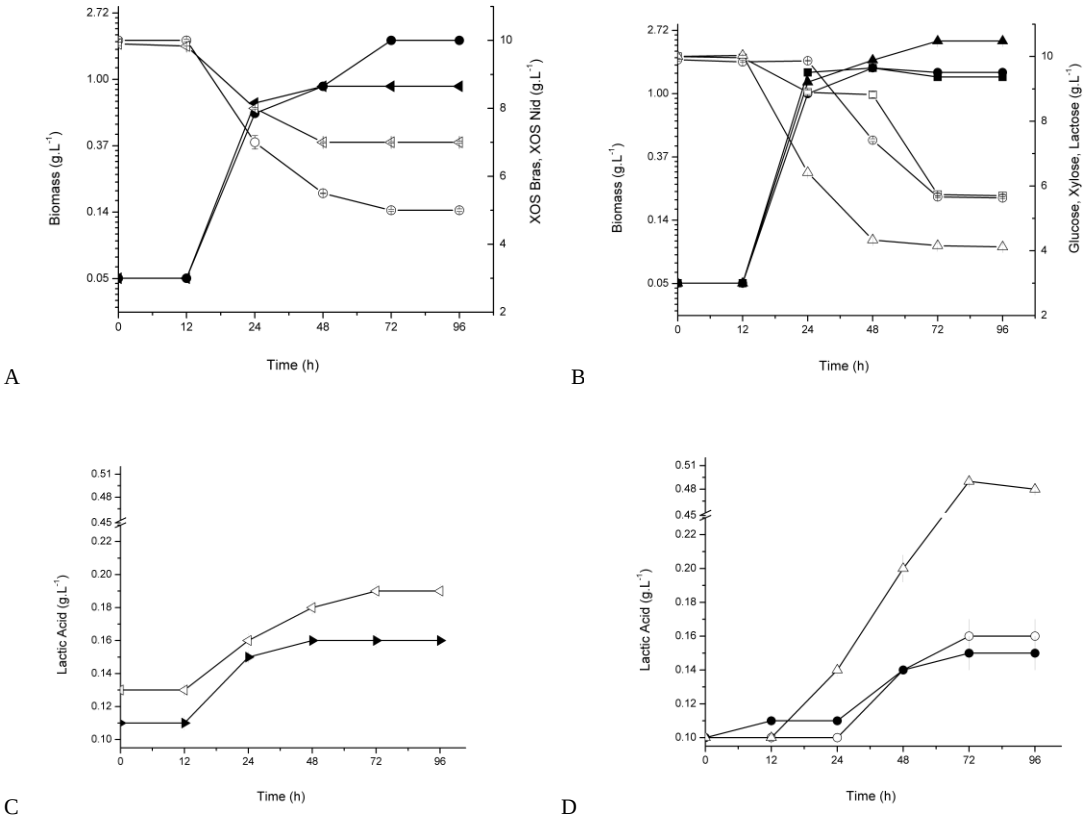


Fig. 4. Growth of *Lactobacillus plantarum* BL011 in different sources of simple sugars and in XOS of enzymatic hydrolysis of rice husk. A) Biomass: (●) XOS *A. brasiliensis*, (◀) XOS *A. nidulans*; and consumption of XOS: (○) XOS *A. brasiliensis*, (◁) XOS *A. nidulans*; B) Biomass: (■) glucose; (●) xylose; and (▲) lactose; and consumption of: (□) glucose; (○) xylose; and (Δ) lactose; C) Production of lactic acid in cultures with XOS: (◁) XOS *A. brasiliensis*; (►) XOS *A. nidulans*; D) Production of lactic acid in cultures with sugars other than XOS: (○) glucose; (●) xylose; and (Δ) lactose. Results are the mean of duplicates.

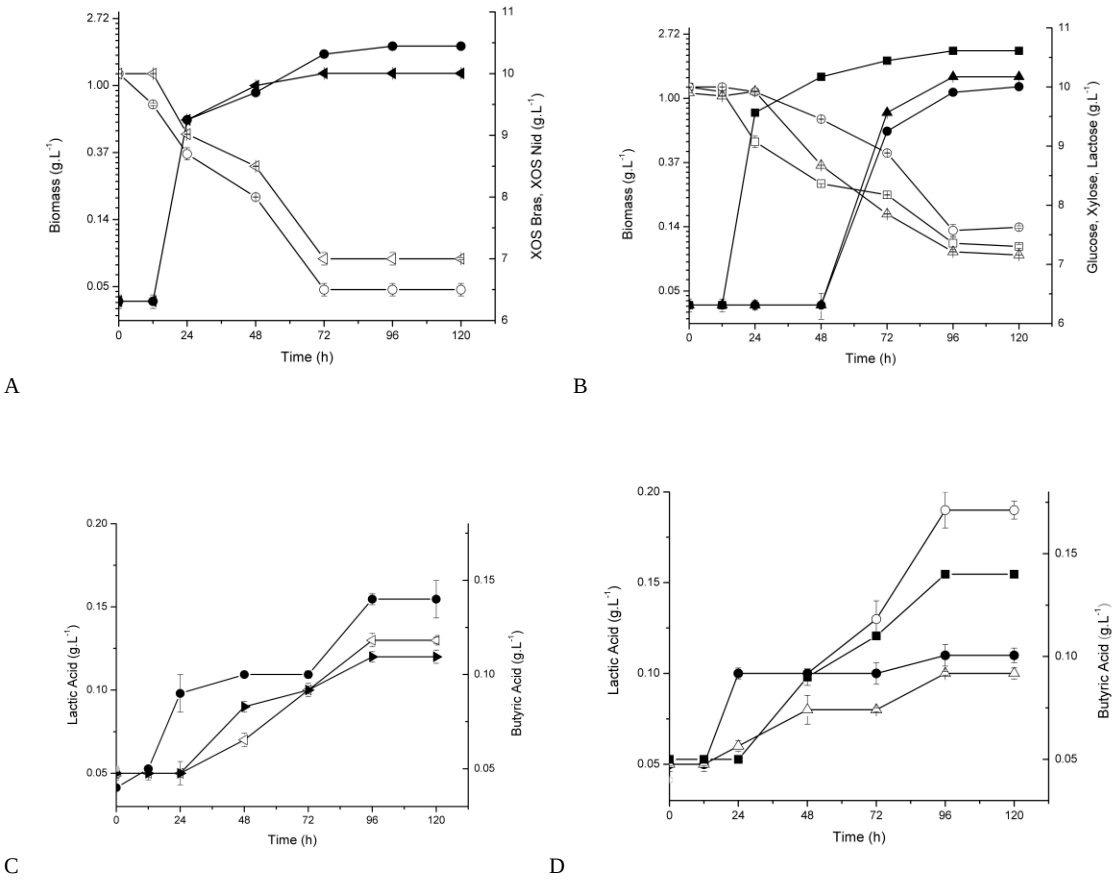


Figure 5. Growth of *Bifidobacterium lactis* BB-12 in different sources of simple sugars and in XOS of enzymatic hydrolysis of rice husk. A) Biomass: (●) XOS *A. brasiliensis*; (◄) XOS *A. nidulans*; and consumption of XOS: (○) XOS *A. brasiliensis*; (◁) XOS *A. nidulans*; B) Biomass: (■) glucose; (●) xylose; (▲) lactose; and consumption of: (□) glucose; (○) xylose; (Δ) lactose); C) Production of lactic acid: (◁) XOS *A. brasiliensis*; (►) XOS *A. nidulans*; and butyric acid: (●) XOS *A. brasiliensis*; D) Production of lactic acid in cultures with sugars other than XOS: (○) glucose; (●) xylose; (Δ) lactose; and butyric acid: (■) glucose. Results are the mean of duplicates.

In an extensive study that investigated the capability of several intestinal and probiotic bacteria to ferment cereal fiber carbohydrates, *L. brevis* was the only

Lactobacilli strain able to grow and metabolize a commercial XOS mixture (Crittenden et al., 2002). In our study, *L. plantarum* BL011 was able to grow in the prepared XOS, reinforcing a cost-effective and value-added process for the production and application of this prebiotic.

According to Mäkeläinen et al. (2010) most *B. lactis* strains studied, among them *B. lactis* BB-12, were able to use commercial XOS to a degree similar to glucose. In our study, *B. lactis* BB-12 growth rate compared well with reports on the literature. *B. adolescentis* NDRI 236 was grown in xylooligosaccharides preparations from agricultural wastes such as Bengal gram husk (BGO) and wheat bran (WBO), with final productions of biomass of 0.04 $g.L^{-1}$ (control, without oligosaccharides), 0.8 $g.L^{-1}$ in BGO, and 0.9 $g.L^{-1}$ in WBO (Madhukumar and Muralikrishna, 2010). In this study, *B lactis* produced a final dry cell mass of 1.7 $g.L^{-1}$ when growing in XOS (Figure 5A).

The three-steps process developed in the present study for XOS production and use as prebiotic has advantages concerning the use of a cheap raw material, simple SSC process, and by using unpurified enzymatic supernatant of xylanase for hydrolysis. In general, both the procedures of biomass pre-treatment and enzyme purification represent high operational costs, which could be avoided using the approaches discussed in this work.

4. Conclusions

We demonstrated the successful production of xylanases in SSC of *Aspergillus brasiliensis* BLf1 and recombinant *Aspergillus nidulans* XynC A773 in rice rusk, a cost-free residue, and used this same biomass to obtain XOS showing degrees of polymerization from 3 to 6. The prebiotic properties of the XOS obtained was proved in

cultures of *Lactobacillus plantarum* BL011 and *Bifidobacterium lactis* BB-12, both showing growth and organic acids production comparable to lactose, their primary sugar in food formulations. Our results support the possibility of scaling-up this process to obtain prebiotics at low costs for functional food applications.

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CAPÍTULO V: DISCUSSÃO GERAL

Após o *screening* obteve-se melhores produções da enzima xilanase com os fungos *Aspergillus brasiliensis* BLf1, *Penicillium crustosum* BLf6, *Penicillium crustosum* BLf7, *Penicillium crustosum* BLf8 e *Aspergillus* sp. BLf11, e a produção máxima foi de 52,41 $U.g^{-1}$ substrato, para fungo *Penicillium crustosum* BLf6 em extrato de malte.

Com o estudo do tipo de cultivo após o *screening*, todos os substratos foram eficazes na indução de atividades enzimáticas, porém a casca de arroz junto ao microrganismo *Aspergillus brasiliensis* BLf1 apresentou as atividades máximas para todas as enzimas, com exceção de celulases. As atividades enzimáticas de xilanase, celulase e β-glicosidase foram geralmente mais elevadas em CES em comparação com CS, produzindo atividades máximas de 120,5, 25,3 e 47,4 $U.g^{-1}$ de substrato, respectivamente.

Após a otimização da produção de xilanase utilizando planejamento experimental em cultivo com *A. brasiliensis* BLf1, conclui-se que as atividades enzimáticas foram influenciadas por variáveis como meio basal, pH e granulometria, sendo a obtenção de máxima atividade de xilanase foi 183,5 $U.g^{-1}$ de substrato para *A. brasiliensis* BLf1. Além disso, foi possível a produção de xilooligossacarídeos com concentrações de 6,41 $mg.g^{-1}$ substrato de X2, 4,49 $mg.g^{-1}$ substrato de X4, 3,58 $mg.g^{-1}$ substrato of X5 e 48,4 $mg.g^{-1}$ substrato de xilose em CES.

No planejamento para a produção da enzima xilanase com o fungo recombinante *Aspergillus nidulans* XynC A773 e *Aspergillus brasiliensis* BLf1 nas mesmas condições de cultivo, obteve-se uma atividade máxima de 230,7 $U.g^{-1}$ para *A. brasiliensis* BLf1 e 187,9 $U.g^{-1}$ para *A. nidulans* XynC A773. As atividades de xilanase foram influenciadas pelas variáveis meio basal e umidade, e também interações entre granulometria e tamanho de inóculo para *A. nidulans* XynC A773.

Os resultados para a produção de xilooligossacarídeos se mostraram efetivos, já que houve uma produção simultânea de XOS junto à produção de xilanase. Ambos os fungos foram capazes de produzir o mesmo perfil de XOS, porém as concentrações variaram. Para *A. brasiliensis* BLf1, se obteve concentrações de 3,37 $mg.g^{-1}$ substrato de X3, 3,18 $mg.g^{-1}$ substrato de X4, 3,75 $mg.g^{-1}$ substrato de X5, 7,33 $mg.g^{-1}$ substrato de X6 e 6,5 $mg.g^{-1}$ substrato de xilose. Enquanto que para *A. nidulans* XynC A773, 6,65

$mg.g^{-1}$ substrato de X3, 6,26 $mg.g^{-1}$ substrato de X4, 5,87 $mg.g^{-1}$ substrato de X5, 5,13 $mg.g^{-1}$ substrato de X6 e 8,18 $mg.g^{-1}$ substrato de xilose.

A aplicação direta do extrato enzimático no substrato, casca de arroz, produziu XOS em concentrações maiores. O perfil de XOS produzido por xilanase de *A. brasiliensis* BLf1 foi: 18,13 $mg.g^{-1}$ substrato de X3, 9,20 $mg.g^{-1}$ substrato de X4, 7,05 $mg.g^{-1}$ substrato de X5, 2,87 $mg.g^{-1}$ substrato de X6 e 68,5 $mg.g^{-1}$ substrate de xilose. Já para o fungo *A. nidulans* XynC A773, as concentrações de XOS foram de 38,57 $mg.g^{-1}$ substrato de X3, 22,19 $mg.g^{-1}$ substrato de X4, 11,40 $mg.g^{-1}$ substrato de X5, 3,76 $mg.g^{-1}$ substrato de X6 e 33.69 $mg.g^{-1}$ substrato de xilose.

Os XOS apresentaram perfil com potencial prebiótico, pois promoveram crescimento de microrganismos probióticos como o *Lactobacillus plantarum* BL011 e *Bifidobacterium Lactis* BB-12. Os XOS como fonte de carbono foram capazes de apresentar um incremento de biomassa destes microrganismos de até 1.7 $g.L^{-1}$.

Desta forma pode-se concluir que o estudo atingiu os objetivos propostos e apresenta interesse biotecnológico, abordando desde a questão ambiental com a utilização de um resíduo agro-industrial, até a elaboração de um produto com valor agregado.

CONCLUSÃO FINAL E SUGESTÕES PARA FUTUROS TRABALHOS

Foi possível utilizar a casca de arroz para produção da enzima xilanase e produção de xilooligossacarídeos por hidrólise enzimática proveniente de cultivo em estado sólido dos fungos filamentosos *Aspergillus brasiliensis* BLf1 e *Aspergillus nidulans* XynC A773. O extrato enzimático de xilanase produzido foi capaz de hidrolisar o resíduo lignocelulósico para produção de xilooligossacarideos com grau de polimerização de 3-6, e este apresentou potencial prebiótico, pois promoveu crescimento de microrganismos probióticos, como *Lactobacillus plantarum* BL011 e *Bifidobacterium Lactis* BB-12.

Podemos concluir que foi possível obter um produto final de baixo custo, alto valor agregado e com possível potencial funcional. Contudo, melhorias ainda podem ser feitas visando aprimorar ainda mais este trabalho. Dessa forma, o avanço nos estudos pode ser realizado a partir de algumas sugestões:

- Realizar o cultivo em biorreator em escala piloto.

- Purificar a enzima e os xilooligossacarídeos.

- Otimizar as condições para a produção de xilooligossacarídeos.

- Realizar testes adicionais necessários para poder afirmar com maior precisão que os xilooligossacarídeos em estudo possui efeito prebiótico.

- Estudar o comportamento dos xilooligossacarídeos produzidos em um produto alimentício.

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